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ATTENTION: BOX PATENT APPLICATION

Sir:

Transmitted herewith for filing is the patent application of

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For: **METHODS OF SCREENING FOR COMPOUNDS THAT MODULATE THE LSR-LEPTIN INTERACTION AND THEIR USE IN THE PREVENTION AND TREATMENT OF OBESITY-RELATED DISEASES**

Enclosed are:

(X) Specification in 110 pages

(X) 54 sheet(s) of drawing.

(X) Sequence Listing in 73 pages

(X) Sequence Listing in computer readable form

(X) Sequence Submission Statement in 1 page

(X) Certificate of Mailing By Express Mail in 1 page

(X) Return prepaid postcard

CLAIMS AS FILED

FOR	NUMBER FILED	NUMBER EXTRA	RATE	FEE
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Total Claims	28 - 20 =	8 ×	$18	$144
Independent Claims	5 - 3 =	2 ×	$78	$156.

If application contains any multiple dependent claims(s), then add		$260	$0
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CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Attorney Docket No.	:	70.US2.REG
Applicant(s)	:	Yen, et al.
For	:	METHODS OF SCREENING FOR COMPOUNDS THAT MODULATE THE LSR-LEPTIN INTERACTION AND THEIR USE IN THE PREVENTION AND TREATMENT OF OBESITY-RELATED DISEASES.
Attorney	:	Heather L. Callahan
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I hereby certify that the accompanying

Application transmittal form in 2 pages; Specification in 110 pages; 54 sheets of drawings; Sequence listing in 73 pages; Sequence listing in computer readable form; Sequence submission statement in 1 page; Return Prepaid Postcard

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Tia de Langen

Tia E. de Langen

METHODS OF SCREENING FOR COMPOUNDS THAT MODULATE THE LSR -LEPTIN INTERACTION AND THEIR USE IN THE PREVENTION AND TREATMENT OF OBESITY-RELATED DISEASES

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 60/155,506 by Yen et al, entitled "Methods of screening for compounds that modulate the LSR-leptin interaction and their use in the prevention and treatment of obesity-related diseases", filed September 22, 1999, which is hereby incorporated by reference herein in its entirety including any figures, drawings or tables.

FIELD OF THE INVENTION

The present invention relates to the field of obesity research, in particular methods of screening for new compounds for the treatment of obesity and obesity-related diseases and disorders, as well as methods of treating obesity-related diseases and disorders. To this end, the characterization of the interaction between a new complex receptor polypeptide, LSR (Lipolysis Stimulated Receptor), and one of its ligands, leptin, is described. The obesity-related diseases or disorders envisaged to be treated by the methods of the invention include, but are not limited to, anorexia, hyperlipidemias, atherosclerosis, diabetes, hypertension and syndrome X. In addition, and more generally, the various pathologies associated with abnormalities in the metabolism of cytokines, may be treated by the methods of the invention.

BACKGROUND OF THE INVENTION

The following discussion is intended to facilitate the understanding of the invention, but is not intended nor admitted to be prior art to the invention.

Obesity is a public health problem that is serious, widespread, and increasing. In the United States, 20 percent of the population is obese; in Europe, a slightly lower percentage is obese (Friedman (2000) Nature 404:632-634). Obesity is associated with increased risk of hypertension, cardiovascular disease, diabetes, and cancer as well as respiratory complications and osteoarthritis (Kopelman (2000) Nature 404:635-643). Even modest weight loss ameliorates these associated conditions.

While still acknowledging that lifestyle factors including environment, diet, age and exercise play a role in obesity, twin studies, analyses of familial aggregation, and adoption studies all indicate that obesity is largely the result of genetic factors (Barsh et al (2000) Nature 404:644-651). In agreement with these studies, is the fact that an increasing number of obesity-related genes are being identified. Some of the more extensively studied genes include those encoding leptin (*ob*) and its receptor (*db*), pro-opiomelanocortin (*Pomc*), melanocortin-4-receptor (*Mc4r*), agouti protein (A^y), carboxypeptidase E (*fat*), 5-hydroxytryptamine receptor 2C (*Htr2c*), nescient basic helix-loop-helix 2 (*Nhlh2*), prohormone convertase 1 IPCSK1), and tubby protein (*tubby*) (rev'd in Barsh et al (2000) Nature 404:644-651).

The gene encoding leptin, one of the most widely studied obesity genes, is involved in the mechanisms of satiety (rev'd in Schwartz et al (2000) Nature 404 :661-671). Leptin is a plasma protein of 16 kDa produced by adipocytes (Zhang et al ((1994) Nature 372:425-432). Mice with an autosomal recessive mutation in this gene (*ob/ob* mice) are obese and hyperphagic. Similarly, mice with an autosomal recessive mutation of the leptin receptor (*db/db* mice, for example) are also obese (Campfield et al (1995) Science 269:546-549). Administration of leptin to *ob/ob,* but not *db/db*, mice corrects their relative hyperphagia and allows normalization of their weight (Weigle (1995) J. Clin. Invest. 96:2065-2070).

Leptin circulates in the body at levels proportional to body fat content(Considine et al (1996) New Eng J Med 334 :292-295) and enters the central nervous system (CNS) at levels proportional to the plasma level (Schwartz et al (1996) Nature Med 2 :589-593). Leptin receptors are expressed by brain neurons involved in energy intake (Baskin et al (1999) Diabetes 48 :828-833; Cheung et al (1997) Endocrinology 138:4489-4492) and administration of leptin into the brain reduces food intake (Weigle (1995) J. Clin. Invest. 96:2065-2070 ; Campfield et al (1995) Science 269:546-549), whereas its deficiency increases food intake (Zhang et al (1994) Nature 372:425-432).

Despite this clear evidence of leptin's role as an adiposity signal, with only a few exceptions the genes encoding leptin or its ob receptor have proved to be normal in obese human subjects (Kopelman et al (2000) Nature 404:635-643). Furthermore, and paradoxically, the plasma concentrations of leptin, are abnormally high in most obese human subjects (Considine et al (1996) New Eng J Med 334 :292-295).

SUMMARY OF THE INVENTION

The present invention results from a focusing of the research effort on the discovery of the mechanisms of leptin elimination. The most widely accepted working hypothesis is that the plasma levels of leptin are high in obese subjects because this hormone is produced by adipose tissue which is increased in obese subjects. In contrast, although not wishing to be limited by any particular theory, the inventors postulated that the concentrations of leptin are increased in obese individuals because the clearance of this hormone is reduced. The resulting high levels of leptin cause a leptin resistance syndrome. Thus, the treatment of obese subjects should not be based on increasing leptin levels, but in normalizing leptin levels.

The lipolysis stimulated receptor (LSR) displays a high affinity for unmodified triglyceride-rich lipoproteins and is involved in the partitioning of dietary lipids among the liver, adipose tissue and muscle. The instant invention stems *inter alia* from studies of the role of LSR in modulating obesity. As part of the instant invention, leptin and the leptin fragment described herein were found to diminish the postprandial lipemic response in db^{Pas}/db^{Pas} mice which lack the leptin OB receptor, thereby showing that leptin signaling can be independent of the OB receptor. Further, the instant invention stems from the discovery that leptin increases the activity of LSR, binds directly to LSR, and that leptin binding leads to leptin degradation. Although not wishing to be bound by a particular theory, the link between leptin

signaling and LSR suggests the post-prandial lipemic response in db^{Pas}/db^{Pas} mice is modulated through this pathway.

In addition, the inventors have discovered that LSR is actually at least two receptors, one for triglyceride-rich lipoproteins, and one for leptin. The three subunits that make up LSR, α, β, and α', actually combine in at least two ways: (1) α and β together make up the LSR receptor for triglyceride-rich lipoproteins, and (2) α' is a necessary part of the LSR receptor for leptin, that may include β as well. Thus, it is now clear that assays can be designed for identifying modulators or receptors/binding partners/signalling cascade members that are specific for the triglyceride-related activity of LSR or for the leptin-related activity of LSR or both.

Further, the invention features the discovery of a 22 amino acid region of human leptin that modulates LSR activity *in vitro* and *in vivo* in the same way as the intact human leptin, thus allowing the use of only this critical region in assays for modulators of the leptin-LSR interaction, and new leptin receptors and binding partners. The new leptin fragment can also be used in disease treatment since it is active in mice at a physiologically-relevant level. In addition, the homologous region from mouse leptin was found to inhibit LSR activity in the human system, and is thus an LSR antagonist of the invention as well as being a powerful tool for identifying further modulators (both inhibitory and stimulatory) of LSR activity.

In a preferred aspect, the invention features a leptin polypeptide fragment that modulates the activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of any one of the leptin polypeptide sequences set forth in Figure 13, wherein said at least 4 and not more than 50 contiguous amaino acids comprise the leptin fragment central sequence. In preferred embodiments, the leptin polypeptide fragment comprises at least 10 but not more than 50, at least 20 but not more than 40, or at least 20 but not more than 30 contiguous amino acids.

Alternatively, the invention features a variant of a leptin polypeptide fragment that modulates the activity of LSR, consisting of a 22 contiguous amino acid sequence that is at least 75% identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13. In preferred embodiments, the variant of a leptin polypeptide fragment is 85% identical, or 95% identical to the leptin fragment variable region. Preferably the leptin fragments and variants are from human or mouse leptin.

In a second aspect, the invention features, a chimeric oligonucleotide, comprising at least 9 contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16, wherein said at least 9 contiguous nucleotides comprise at least one amino acid codon selected from the group consisting of TTA, TTG, TCA, TCG, TAU, TAC, TGT, TGC, TGG, CAA, CAG, AGA, GAA, GAG, and GGA, and wherein a point mutation is present in said codon such that said codon is a stop codon. Alternatively, the chimeric oligonucleotide comprises at least 9

contiguous nucleotides of SEQ ID NO:1, wherein said at least 9 contiguous nucleotides comprise a single nucleotide polymorphism selected from the group consisting of A1 to A32.

In a third aspect, the invention features a zinc finger protein, comprising a DNA binding domain that binds specifically to 18 nucleotides of a sequence at least 50% homologous to SEQ ID NO:1, wherein said 18 nucleotides comprise two fragments of 9 contiguous nucleotides, and wherein said fragments are separated by 0, 1, 2, or 3 nucleotides. In preferred embodiments, said sequence is at least 50% homologous to intronic sequences selected from the group consisting of 2357 to 3539, 3885 to 12162, 12283 to 15143, 15201 to 17764, 15912 to 19578, 19753 to 19898, 19959 to 20055, 20188 to 20328, and 20958 to 21046 of SEQ ID NO:1, preferably to residues 2357 to 3539 of SEQ ID NO:1, or alternatively 5' untranslated regions such as the sequence 1 to 2356 of SEQ ID NO:1. In preferred embodiments, said protein further comprises a functional domain selected from the group consisting of a transcription repressor and a transcription initiator; preferably said repressor is a KRAB repressor and said initiator is a VP16 initiator. In other preferred embodiments, said protein further comprises a small molecule regulatory system, preferably said system is selected from the group consisting of a Tet system, RU486, and ecdysone.

In a fourth aspect, the invention features polynucleotides encoding the leptin polypeptide fragments and variants of the invention, or polynucleotides encoding a zinc finger protein of the invention.

In a fifth aspect, the invention features recombinant vectors comprising the polynucleotides encoding the leptin polypeptide fragments and variants of the invention, or polynucleotides or recombionant vectors encoding a zinc finger protein of the invention. In preferred embodiments, said vector is an adenovirus associated virus.

In a sixth aspect, the invention features recombinant cells comprising the polynucleotides and recombinant vectors encoding the leptin polypeptide fragments and variants of the invention, or polynucleotides and recombinant vectors encoding zinc finger proteins of the invention. In preferred embodiments, the recombinant cell comprising the polynucleotides and recombinant vectors encoding leptin fragments and variants and zinc finger polypeptides of the invention, are transfected with at least one LSR polypeptide comprising a sequence at least 75% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19. Preferably, said transfected cell is stably transfected. Preferably, said cell is selected from the group consisting of PLC, CHO-K1, Hep3B, Hepa 1-6, and HepG2.

In a seventh embodiment, the invention features a pharmaceutical composition comprising the leptin polypeptide fragments and variants of the invention, or polynucleotides or recombinant vectors encoding a zinc finger protein of the invention, or chimeraplasts of the invention.

In an eighth aspect, the invention features non-human mammals comprising polynucleotides and recombinant vectors encoding zinc finger proteins of the invention. Preferably, said vector is an adenovirus associated virus.

In a ninth aspect, the invention features a method of treating or preventing an obesity-related disease or disorder comprising providing to an individual in need of such treatment a pharmaceutical composition comprising the leptin polypeptide fragments and variants of the invention. Preferably, said disease is congenital generalized lipodystrophy. Alternatively, the patient is provided a chimeric oligonucleotide of the invention or a polynucleotide or recombinant vector encoding a zinc finger protein of the invention. Preferably, said providing comprises a liposome, and preferably said vector is an adenovirus associated virus. In preferred embodiments, the obesity related disease or disorder is selcted from the group consisting of obesity, anorexia, cachexia, cardiac insufficiency, coronary insufficiency, stroke, hypertenison, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, hyperuricemia, and Syndrome X. Preferably the individual is an animal, preferably a mammal, most preferably a human.

In a tenth aspect, the invention features a method of designing mimetics of a leptin fragment that modulates an activity of LSR, comprising: identifying critical interactions between one or more amino acids of said leptin fragment and LSR; designing potential mimetics to comprise said critical interactions; and testing said potential mimetics ability to modulate said activity as a means for designing said mimetics. Preferably, the leptin fragment consists of the leptin fragment variable region or the leptin fragment central sequence of any one of the leptin polypeptide sequences set forth in Figure 13. Alternatively, the leptin fragment is any one of the leptin fragments or variants of the invention. Preferably, the leptin fragment or variant is from human or mouse leptin. In preferred embodiments, the activity of lSR is selected from the group consisting of leptin binding, leptin uptake, leptin degradation, triglyceride binding, triglyceride uptake, and triglyceride degradation. Preferably the critical interactions are selected from the group consisting of hydrogen bonding, covalent bonding, Van der Waals forces, steric hindrances, and hydrophobic interactions, and are identified using assays selected from the group consisting of NMR, X-ray crystallography, and computer modeling.

In an eleventh aspect, the invention features a method of inhibiting the expression of at least one subunit of LSR, comprising providing to a cell a chimeric oligonucleotide of the invention that changes a amino acid codon to a stop codon. Preferably, the cell is selected from the group consisting of PLC, CHO-K1, HepG2, Hepa 1-6, and Hep3B. Alternatively the cell is in a mammal, preferably a mouse, more preferably in a human, and is provided using a liposome.

In a related aspect, the invention features a method of modulating the expression of at least one subunit of LSR, comprising providing to a cell a polynucleotide encoding a zinc finger protein of the invention. Preferably, said cell is selected from the group consisting of PLC, CHO-K1, HepG2, Hepa 1-

6, and Hep3B. Alternatively, said cell is in an animal, preferably a mammal, and preferably said mammal is a mouse or a human.

In a twelfth aspect, the invention features a method for selecting a compound useful for the treatment or prevention of an obesity-related disease or disorder, comprising: contacting a recombinant cell comprising a polynucleotide or recombinant vector encoding a zinc finger protein of the invention, and that optionally further comprises at least one LSR polypeptide comprising a sequence at least 75% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19, with a candidate compound; and detecting a result selected from the group consisting of a modulation of an activity of the Lipolysis Stimulated Receptor and modulation of expression of the Lipolysis Stimulated Receptor; as a means for selecting said compound useful for the treatment or prevention of said obesity-related disease or disorder. In preferred embodiments, said contacting is in the presence of a ligand of said Lipolysis Stimulated Receptor. Preferably, said ligand is selected from the group consisting of cytokine, lipoprotein, free fatty acids, Apm1, and C1q. Most preferably said cytokine is leptin, or a leptin polypeptide fragment or variant of the invention. Alternatively said free fatty acid is oleate.

In preferred embodiments, said LSR activity is selected from the group consisting of binding of lipoproteins, uptake of lipoproteins, degradation of lipoproteins, binding of leptin, uptake of leptin, and degradation of leptin. Preferably said modulation is an increase in said activity, alternatively a decrease in activity. In other preferred embodiments, said expression is on the surface of said cell, and preferably said detecting comprises FACS. Preferably, said detecting further comprises antibodies that bind specifically to said LSR, wherein said LSR comprises an amino acid sequence at least 75% homologous to at least one of the sequences selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19 Most preferably, said antibodies bind specifically to a region of said LSR selected from the group consisting of an amino terminus, a carboxy terminus, a splice site, a cytokine binding site, a fatty acid binding site, a clathrin binding site, an apoprotein ligand binding site, a LI/LL motif, a RSRS motif, and a hydrophobic region. Preferably, said cell is selected from the group consisting of PLC, CHO-K1, Hep3B, Hepa 1-6, and HepG2.

In other preferred embodiments, said candidate compound is selected from the group consisting of peptides, peptide libraries, non-peptide libraries, peptoids, fatty acids, lipoproteins, medicaments, antibodies, and small molecules. Preferably, said obesity-related diseases and disorders are selected from the group consisting of obesity, anorexia, cachexia, cardiac insufficiency, coronary insufficiency, stroke, hypertension, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, hyperuricemia, and Syndrome X.

In a thirteenth aspect, the invention features a method of selecting for genes that modulate an activity of the Lipolysis Stimulated Receptor, comprising : providing a retroviral gene library to cells that express said Lipolysis Stimulated Receptor; contacting said cells with a ligand of said Lipolysis Stimulated Receptor ; detecting a change in said activity of the Lipolysis Stimulated Receptor as a means for selecting for said genes. In preferred embodiments, said retroviral gene library comprises a cDNA library from tissues selected from the group consisting of liver and adipose. Preferably, said retroviral gene library further comprises a detectable marker protein selected from the group consisting of GFP, truncated CD2, and truncated CD4. In other preferred embodiments, the invention further comprises selecting said cells comprising the retroviral gene library for moderate expression of GFP; preferably said selecting of cells is by FACS.

In other preferred embodiments, said ligand is selected from the group consisting of cytokine, lipoprotein, free fatty acids, Apm1, and C1q. Most preferably said cytokine is leptin, or a leptin polypeptide fragment or variant of the invention. Alternatively said free fatty acid is oleate.

In yet other preferred embodiments, preferably said detecting a change in said activity comprises FACS. Preferably, said detecting further comprises antibodies that bind specifically to said LSR, wherein said LSR comprises an amino acid sequence at least 75% homologous to at least one of the sequences selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19 Most preferably, said antibodies bind specifically to a region of said LSR selected from the group consisting of an amino terminus, a carboxy terminus, a splice site, a cytokine binding site, a fatty acid binding site, a clathrin binding site, an apoprotein ligand binding site, a LI/LL motif, a RSRS motif, and a hydrophobic region. Preferably, said cell is selected from the group consisting of PLC, CHO-K1, Hep3B, Hepa 1-6, and HepG2.

DETAILED DESCRIPTION OF THE INVENTION

LSR (Lipolysis Stimulated Receptor), which is described in PCT publication No WO IB98/01257 (hereby incoporated by reference herein in its entirety including any figures, tables, or drawings), is expressed on the surface of hepatic cells, and is involved in the partitioning of dietary lipids between the liver and peripheral tissues, including muscles and adipose tissue. The *LSR* gene encodes, by alternative splicing, three types of subunits, LSR α, LSR α', and LSR β. The α' subunit specifically binds a cytokine, leptin, which activates LSR and is taken up and degraded. The invention is drawn *inter alia* to compounds that modulate the interaction between LSR and leptin useful in the treatment or prevention of obesity-related diseases and disorders.

Definitions

Before describing the invention in greater detail, the following definitions are set forth to illustrate and define the meaning and scope of the terms used to describe the invention herein.

As used interchangeably herein, the terms "oligonucleotides", and "polynucleotides" include RNA, DNA, or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form. The terms "nucleotide", "nucleotide sequence" and "nucleic acid" are used herein consistently with their use in the art, including to encompass "modified nucleotides" which comprise at least one modification, including by way of example and not limitation: (a) an alternative linking group, (b) an analogous form of purine, (c) an analogous form of pyrimidine, or (d) an analogous sugar. For examples of analogous linking groups, purines, pyrimidines, and sugars see for example PCT publication No. WO 95/04064. The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, *ex vivo* generation, or a combination thereof, as well as utilizing any purification methods known in the art.

The terms polynucleotide construct, recombinant polynucleotide and recombinant polypeptide are used herein consistently with their use in the art. The terms "upstream" and "downstream" are also used herein consistently with their use in the art. The terms "base paired" and "Watson & Crick base paired" are used interchangeably herein and consistently with their use in the art. Similarly, the terms "complementary", "complement thereof" , "complement", "complementary polynucleotide", "complementary nucleic acid" and "complementary nucleotide sequence" are used interchangeably herein and consistently with their use in the art.

The term "purified" is used herein to describe a polynucleotide or polynucleotide vector of the invention that has been separated from other compounds including, but not limited to, other nucleic acids, carbohydrates, lipids and proteins (such as the enzymes used in the synthesis of the polynucleotide). Purified can also refer to the separation of covalently closed polynucleotides from linear polynucleotides, or vice versa, for example. A polynucleotide is substantially pure when at least about 50%, 60%, 75%, or 90% of a sample contains a single polynucleotide sequence. In some cases this involves a determination between conformations (linear versus covalently closed). A substantially pure polynucleotide typically comprises about 50, 60, 70, 80, 90, 95, 99% weight/weight of a nucleic acid sample. Polynucleotide purity or homogeneity may be indicated by a number of means well known in the art, such as agarose or polyacrylamide gel electrophoresis of a sample, followed by visualizing a single polynucleotide band upon staining the gel. For certain purposes higher resolution can be provided by using HPLC or other means well known in the art.

Similarly, the term "purified" is used herein to describe a polypeptide of the invention that has been separated from other compounds including, but not limited to, nucleic acids, lipids, carbohydrates and other proteins. In some preferred embodiments, a polypeptide is substantially pure when at least about 50%, 60%, 75%, 85%, 90%, or 95% of a sample exhibits a single polypeptide sequence. In some

preferred embodiments, a substantially pure polypeptide typically comprises about 50%, 60%, 70%, 80%, 90% 95%, or 99% weight/weight of a protein sample. Polypeptide purity or homogeneity is indicated by a number of methods well known in the art, such as agarose or polyacrylamide gel electrophoresis of a sample, followed by visualizing a single polypeptide band upon staining the gel. For certain purposes higher resolution can be provided by using HPLC or other methods well known in the art.

Further, as used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. Alternatively, purification may be expressed as "at least" a percent purity relative to heterologous polynucleotides (DNA, RNA or both) or polypeptides. As a preferred embodiment, the polynucleotides or polypeptides of the present invention are at least; 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% pure relative to heterologous polynucleotides or polypeptides. As a further preferred embodiment the polynucleotides or polypeptides have an "at least" purity ranging from any number, to the thousandth position, between 90% and 100% (e.g., at least 99.995% pure) relative to heterologous polynucleotides or polypeptides. Additionally, purity of the polynucleotides or polypeptides may be expressed as a percentage (as described above) relative to all materials and compounds other than the carrier solution. Each number, to the thousandth position, may be claimed as individual species of purity.

The term "isolated" requires that the material be removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that the vector or composition is not part of its natural environment.

Specifically excluded from the definition of "isolated" are: naturally occurring chromosomes (*e.g.*, chromosome spreads), artificial chromosome libraries, genomic libraries, and cDNA libraries that exist either as an *in vitro* nucleic acid preparation or as a transfected/transformed host cell preparation, wherein the host cells are either an *in vitro* heterogeneous preparation or plated as a heterogeneous population of single colonies. Also specifically excluded are the above libraries wherein a 5' EST makes up less than 5% of the number of nucleic acid inserts in the vector molecules. Further specifically excluded are whole cell genomic DNA or whole cell RNA preparations (including said whole cell preparations which are mechanically sheared or enzymatically digested). Further specifically excluded are the above whole cell preparations as either an *in vitro* preparation or as a heterogeneous mixture separated by electrophoresis (including blot transfers of the same) wherein the polynucleotide of the invention have not been further

separated from the heterologous polynucleotides in the electrophoresis medium (*e.g.*, further separating by excising a single band from a heterogeneous band population in an agarose gel or nylon blot).

The term "primer" denotes a specific oligonucleotide sequence which is complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by DNA polymerase, RNA polymerase, or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, e.g., PNA as defined hereinbelow) which can be used to identify a specific polynucleotide sequence present in a sample, said nucleic acid segment comprising a nucleotide sequence complementary to the specific polynucleotide sequence to be identified.

The term "polypeptide" refers to a polymer of amino acids without regard to the length of the polymer. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude post-expression modifications of polypeptides. For example, polypeptides that include the covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like are expressly encompassed by the term polypeptide. Also included within the definition are polypeptides which contain one or more analogs of an amino acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

Without being limited by theory, the compounds/polypeptides of the invention are believed to treat "diseases involving the partitioning of dietary lipids between the liver and peripheral tissues ". The term "peripheral tissues" is meant to include muscle and adipose tissue. In preferred embodiments, the compounds/polypeptides of the invention partition the dietary lipids toward the muscle. In alternative preferred embodiments, the dietary lipids are partitioned toward the adipose tissue. In other preferred embodiments, the dietary lipids are partitioned toward the liver. In yet other preferred embodiments, the compounds/polypeptides of the invention increase or decrease the oxidation of dietary lipids, preferably free fatty acids (FFA) by the muscle. Dietary lipids include, but are not limited to triglycerides and free fatty acids.

Preferred diseases believed to involve the partitioning of dietary lipids include obesity and obesity-related diseases and disorders such as atherosclerosis, heart disease, insulin resistance, hypertension, stroke, Syndrome X, and Type II diabetes. Type II diabetes-related complications to be treated by the methods of the invention include microangiopathic lesions, ocular lesions, and renal lesions. Heart disease includes, but is not limited to, cardiac insufficiency, coronary insufficiency, and high blood pressure. Other obesity-related disorders to be treated by compounds of the invention include hyperlipidemia and hyperuricemia. Yet other obesity-related diseases or disorders of the invention

include cachexia, wasting, AIDS-related weight loss, neoplasia-related weight loss, anorexia, and bulimia.

The term "obesity" as used herein is defined in the WHO classifications of weight (Kopelman (2000) Nature 404:635-643). Underweight is less than 18.5 (thin); Healthy is 18.5-24.9 (normal); grade 1 overweight is 25.0-29.9 (overweight); grade 2 overweight is 30.0-39.0 (obesity); grade 3 overweight is greater than or equal to 40.0 BMI (morbid obesity). BMI is body mass index and is kg/m^2. Waist circumference can also be used to indicate a risk of metabolic complications where in men a circumference of greater than or equal to 94 cm indicates an increased risk, and greater than or equal to 102 cm indicates a substantially increased risk. Similarly for women, greater than or equal to 88 cm indicates an increased risk, and greater than or equal to 88 cm indicates a substantially increased risk. The waist circumference is measured in cm at midpoint between lower border of ribs and upper border of the pelvis. Other measures of obesity include, but are not limited to, skinfold thickness which is a measurement in cm of skinfold thickness using calipers, and bioimpedance, which is based on the principle that lean mass conducts current better than fat mass because it is primarily an electrolyte solution; measurement of resistance to a weak current (impedance) applied across extremities provides an estimate of body fat using an empirically derived equation.

The term "agent acting on the partitioning of dietary lipids between the liver and peripheral tissues" refers to a compound or polypeptide of the invention that modulates the partitioning of dietary lipids between the liver and the peripheral tissues as previously described. Preferably, the agent increases or decreases the oxidation of dietary lipids, preferably free fatty acids (FFA) by the muscle. Preferably the agent decreases or increases the body weight of individuals or is used to treat or prevent an obesity-related disease or disorder such as atherosclerosis, heart disease, insulin resistance, hypertension, stroke, Syndrome X, and Type II diabetes. Type II diabetes-related complications to be treated by the methods of the invention include, but are not limited to, microangiopathic lesions, ocular lesions, and renal lesions. Heart disease includes, but is not limited to, cardiac insufficiency, coronary insufficiency, and high blood pressure. Other obesity-related disorders to be treated by compounds of the invention include hyperlipidemia and hyperuricemia. Yet other obesity-related diseases or disorders of the invention include cachexia, wasting, AIDS-related weight loss, anorexia, and bulimia.

The terms "response to an agent acting on the partitioning of dietary lipids between the liver and peripheral tissues " refer to drug efficacy, including but not limited to, ability to metabolize a compound, to the ability to convert a pro-drug to an active drug, and to the pharmacokinetics (absorption, distribution, elimination) and the pharmacodynamics (receptor-related) of a drug in an individual.

The terms "side effects to an agent acting on the partitioning of dietary lipids between the liver and peripheral tissues " refer to adverse effects of therapy resulting from extensions of the principal pharmacological action of the drug or to idiosyncratic adverse reactions resulting from an interaction of the drug with unique host factors. "Side effects to an agent acting on the partitioning of dietary lipids

between the liver and peripheral tissues " can include, but are not limited to, adverse reactions such as dermatologic, hematologic or hepatologic toxicities and further includes gastric and intestinal ulceration, disturbance in platelet function, renal injury, nephritis, vasomotor rhinitis with profuse watery secretions, angioneurotic edema, generalized urticaria, and bronchial asthma to laryngeal edema and bronchoconstriction, hypotension, and shock.

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one binding domain, where an antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, and that allows an immunological reaction with the antigen. Antibodies include recombinant proteins comprising the antibody binding domains, as well as fragments, including Fab, Fab', $F(ab)_2$, and $F(ab')_2$ fragments.

As used herein, an "antigenic determinant" is the portion of an antigen molecule, in this case an LSR polypeptide, that determines the specificity of the antigen-antibody reaction. An "epitope" refers to an antigenic determinant of a polypeptide. An epitope can comprise as few as 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 6 such amino acids, and more usually at least 8-10 such amino acids. Methods for determining the amino acids which make up an epitope include x-ray crystallography, 2-dimensional nuclear magnetic resonance, and epitope mapping e.g. the Pepscan method described by H. Mario Geysen et al. 1984. Proc. Natl. Acad. Sci. U.S.A. 81:3998-4002; PCT Publication No. WO 84/03564; and PCT Publication No. WO 84/03506.

The term "compound" as used herein refers to molecules, either organic or inorganic, that can be tested for activity in an assay. Preferably, compounds have a low molecular weight of less than 500 kda, some compounds can have a molecular weight between 500 and 1500, other compounds may have a molecular weight of at least 1500 kda. In addition, compounds of interest preferably have a desired activity at a low concentration, *e.g.* a compound that is active at a concentration of 1 ng/mL or less, is generally preferred over one that is active at 1 ng/mL to 100 ng/mL, or one that is active only at concentrations greater than 100 ng/mL. Examples of compounds to be tested in the assays herein include: peptides, peptide libraries, non-peptide libraries, antibodies, and peptoids.

The term "activity" as used herein refers to a measurable result of the interaction of molecules. For example, some LSR activities include leptin binding, leptin uptake, leptin degradation, as well as triglyceride binding, triglyceride uptake, and triglyceride degradation. Some exemplary methods of measuring these activities are provided herein.

The term "modulate" as used herein refers to the ability of a compound to change an activity in some measurable way as compared to an appropriate control. As a result of the presence of compounds in the assays, activities can increase (*e.g.* there could be increased levels of leptin binding), or "decrease" (*e.g.* there could be decreased levels of leptin binding) as compared to controls in the absence of these

compounds. Preferably, an increase in activity is at least 25%, more preferably at least 50%, most preferably at least 100% compared to the level of activity in the absence of the compound. Similarly, a decrease in activity is preferably at least 25%, more preferably at least 50%, most preferably at least 100% compared to the level of activity in the absence of the compound. A compound that increases a known activity is an "agonist". One that decreases, or prevents, a known activity is an "antagonist".

The term "monitoring" as used herein refers to any method in the art by which an activity can be measured. For each of the activities in the assays of the invention, exemplary methods are provided in the Examples section.

The term "providing" as used herein refers to any means of adding a compound or molecule to something known in the art. Examples of providing can include the use of pipets, pipettmen, syringes, needles, tubing, guns, etc. This can be manual or automated. It can include transfection by any mean or any other means of providing nucleic acids to dishes, cells, tissue, cell-free systems and can be *in vitro* or *in vivo*. Methods are provided in the Examples section as examples.

The term "LSR-related diseases and disorders" as used herein refers to any disease or disorder or condition comprising an aberrant functioning of LSR, or a subunit(s) of LSR, to include aberrant levels of expression of LSR, or a subunit(s) of LSR (either increased or decreased), aberrant activity of LSR (either increased or decreased), and aberrant interactions with ligands or binding partners (either increased or decreased). By "aberrant" is meant a change from the type, or level of activity seen in normal cells, tissues, or individuals, or seen previously in the cell, tissue, or individual prior to the onset of the illness.

The term "cosmetic treatments" is meant to include treatments with compounds or polypeptides of the invention that increase or decrease the body mass of an individual where the individual is not clinically obese or clinically thin. Thus, these individuals have a body mass index (BMI) below the cut-off for clinical obesity (*e.g.* below 25 kg/m^2) and above the cut-off for clinical thinness (*e.g.* above 18.5 kg/m^2). In addition, these individuals are preferably healthy (*e.g.* do not have an obesity-related disease or disorder of the invention). "Cosmetic treatments" are also meant to encompass, in some circumstances, more localized increases in adipose tissue, for example, gains or losses specifically around the waist or hips, or around the hips and thighs, for example. These localized gains or losses of adipose tissue can be identified by increases or decreases in waist or hip size, for example.

The term "preventing" as used herein refers to administering a compound prior to the onset of clinical symptoms of a disease or conditions so as to prevent a physical manifestation of aberrations associated with obesity or LSR.

The term "treating" as used herein refers to administering a compound after the onset of clinical symptoms.

The term "in need of treatment" as used herein refers to a judgment made by a caregiver (*e.g.* physician, nurse, nurse practitioner, etc in the case of humans; veterinarian in the case of animals,

including non-human mammals) that an individual or animal requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a care giver's expertise, but that include the knowledge that the individual or animal is ill, or will be ill, as the result of a condition that is treatable by the compounds of the invention.

The term "perceives a need for treatment" refers to a sub-clinical determination that an individual desires to reduce weight for cosmetic reasons as discussed under "cosmetic treatment" above. The term "perceives a need for treatment" in other embodiments can refer to the decision that an owner of an animal makes for cosmetic treatment of the animal.

The term "individual" as used herein refers to a mammal, including animals, preferably mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, most preferably humans.

The term "non-human animal" refers to any non-human vertebrate, birds and more usually mammals, preferably primates, animals such as swine, goats, sheep, donkeys, horses, cats, dogs, rabbits or rodents, more preferably rats or mice. Both the terms "animal" and "mammal" expressly embrace human subjects unless preceded with the term "non-human".

The terms "percentage of sequence identity" and "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Homology is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85(8):2444-2448; Altschul et al., 1990, J. Mol. Biol. . 215(3):403-410) Thompson et al., 1994, Nucleic Acids Res. 22(2):4673-4680; Higgins et al., 1996, Methods Enzymol. 266:383-402; Altschul et al., 1990, J. Mol. Biol. 215(3):403-410; Altschul et al., 1993, Nature Genetics 3:266-272). In a particularly preferred embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") which is well known in the art (see e.g., Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2267-2268; Altschul et al., 1990, J. Mol. Biol. 215:403-410; Altschul et al., 1993, Nature Genetics 3:266-272; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402. In particular, five specific BLAST programs are used to perform the following task:

(1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;

(2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;

(3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database;

(4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and

(5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet et al., 1992, Science 256:1443-1445; Henikoff and Henikoff, 1993, Proteins 17:49-61. Less preferably, the PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure, Washington: National Biomedical Research Foundation). The BLAST programs evaluate the statistical significance of all high-scoring segment pairs identified, and preferably selects those segments which satisfy a user-specified threshold of significance, such as a user-specified percent homology. Preferably, the statistical significance of a high-scoring segment pair is evaluated using the statistical significance formula of Karlin (see, e.g., Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2267-2268).

By way of example and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/mL denatured salmon sperm DNA. Filters are hybridized for 48 h at 65 °C, the preferred hybridization temperature, in prehybridization mixture containing 100 µg/mL denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Alternatively, the hybridization step can be performed at 65 °C in the presence of SSC buffer, 1 x SSC corresponding to 0.15 M NaCl and 0.05 M Na citrate. Subsequently, filter washes can be done at 37 °C for 1 h in a solution containing 2 x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA, followed by a wash in 0.1 X SSC at 50 °C for 45 min. Alternatively, filter washes can be performed in a solution containing 2 x SSC and 0.1% SDS, or 0.5 x SSC and 0.1% SDS, or 0.1 x SSC and 0.1% SDS at 68 °C for 15 minute intervals. Following the wash steps, the hybridized probes are detectable by autoradiography. Other conditions of high stringency that may be used are well known in the art (see, for example, Sambrook et al., 1989; and Ausubel et al., 1989, both of which are hereby incorporated by reference herein in their entirety). These hybridization conditions are suitable for a nucleic acid molecule of about 20 nucleotides in length. A person of ordinary skill in the art will realize that the hybridization conditions described

above are to be adapted according to the length of the desired nucleic acid following techniques well known to the one skilled in the art. Suitable hybridization conditions may for example be adapted according to the teachings disclosed in the book of Hames and Higgins (1985) or in Sambrook et al.(1989).

Variants

It will be recognized by one of ordinary skill in the art that some amino acids of the polypeptide sequences of the present invention can be varied without significant effect on the structure or function of the protein; there will be critical amino acids in the polypeptide sequence that determine activity. Thus, the invention further includes variants of polypeptides. Such variants include polypeptide sequences with one or more amino acid deletions, insertions, inversions, repeats, and substitutions either from natural mutations or human manipulation selected according to general rules known in the art so as to have little effect on activity. Guidance concerning how to make phenotypically silent amino acid substitutions is provided below.

There are two main approaches for studying the tolerance of an amino acid sequence to change (*See*, Bowie, J. U. et al. 1990). The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality.

These studies have revealed that proteins are surprisingly tolerant of amino acid substitutions and indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described by Bowie et al. (supra) and the references cited therein.

Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Phe; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe, Tyr. In addition, the following groups of amino acids generally represent equivalent changes: (1) Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr; (2) Cys, Ser, Tyr, Thr; (3) Val, Ile, Leu, Met, Ala, Phe; (4) Lys, Arg, His; (5) Phe, Tyr, Trp, His.

Similarly, amino acids in polypeptide sequences of the invention that are essential for function can also be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (*See, e.g.*, Cunningham et al. 1989). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for obesity-related activity using assays as described above. Of special interest are substitutions of charged amino acids with other charged or neutral amino acids that may produce proteins with highly desirable improved

characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because aggregates can be immunogenic, (*See, e.g.*, Pinckard, et al., 1967; Robbins, et al., 1987;and Cleland, et al., 1993).

Thus, the fragment, derivative, analog, or homolog of the polypeptide of the present invention may be, for example: (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code: or (ii) one in which one or more of the amino acid residues includes a substituent group: or (iii) one in which the polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol): or (iv) one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the above form of the polypeptide or a pro-protein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of a polypeptide having an amino acid sequence which contains at least one conservative amino acid substitution, but not more than 50 conservative amino acid substitutions, not more than 40 conservative amino acid substitutions, not more than 30 conservative amino acid substitutions, and not more than 20 conservative amino acid substitutions. Also provided are polypeptides which comprise the amino acid sequence of polypeptide, having at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 conservative amino acid substitutions.

Another specific embodiment of a modified polypeptide of the invention is a polypeptide that is resistant to proteolysis, for example a polypeptide in which a -CONH- peptide bond is modified and replaced by one or more of the following: a (CH2NH) reduced bond; a (NHCO) retro inverso bond; a (CH2-O) methylene-oxy bond; a (CH2-S) thiomethylene bond; a (CH2CH2) carba bond; a (CO-CH2) cetomethylene bond; a (CHOH-CH2) hydroxyethylene bond); a (N-N) bound; a E-alcene bond; or a -CH=CH- bond. Thus, the invention also encompasses a polypeptide or a fragment or a variant thereof in which at least one peptide bond has been modified as described above.

In addition, amino acids have chirality within the body of either L or D. In some embodiments it is preferable to alter the chirality of the amino acids in the polypeptides of the invention in order to extend half-life within the body. Thus, in some embodiments, one or more of the amino acids are preferably in the L configuration. In other embodiments, one or more of the amino aicds are preferably in the D configuration.

I. Leptin Polynucleotides of the Invention

Polynucleotides have been designed that encode a LSR-binding/activating/modulating portion of the leptin protein. This region was identified by a comparison of the human and murine amino acid sequences, and its activity was confirmed *in vitro* and *in vivo* (See Examples 1-8). The recombinant polynucleotide encoding the LSR-activating leptin fragment can be used in a variety of ways, including: (1) to express the polypeptide in recombinant cells so as to be purified and used as described below, (2) to express the polypeptide in cells as part of an assay system to discover modulators of the leptin/LSR interaction, and (3) as part of a gene surgery where the fragment itself can be used in treatment and/or prevention of obesity-related diseases and disorders and modulating body mass.

The invention relates to the polynucleotides encoding a leptin polypeptide fragment described in the Examples (7 & 8), and variants and fragments thereof as described herein in Leptin Polypeptides of the Invention (section II), as well as to variants and fragments of the polynucleotides that encode these polypeptides. Preferably, polynucleotides are purified, isolated and/or recombinant.

In other preferred embodiments, variants of the leptin polynucleotides encoding leptin polypeptides as described herein in Leptin Polypeptides of the Invention are envisioned. Variants of polynucleotides, as the term is used herein, are polynucleotides whose sequence differs from a reference polynucleotide. A variant of a polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical.

Variants of leptin polynucleotides according to the invention may include, without being limited to, nucleotide sequences which are at least 90% (preferably at least 95%, more preferably at least 99%, and most preferably at least 99.5%) identical to a polynucleotide that encodes a leptin polypeptide of the invention, or to any polynucleotide fragment of at least 8 (preferably at least 15, more preferably at least 25, and most preferably at least 45) consecutive nucleotides of a polynucleotide that encodes a polypeptide of the invention.

Nucleotide changes present in a variant polynucleotide are preferably silent, which means that they do not alter the amino acids encoded by the polynucleotide. However, nucleotide changes may also result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. The substitutions, deletions or additions may involve one or more nucleotides. Alterations in the leptin coding regions of the invention may produce conservative or non-conservative amino acid substitutions, deletions or additions in the encoded protein. Preferably, the nucleotide substitutions result in non-conservative amino acid changes and more preferably in conservative amino acid changes in the encoded polypeptide.

In cases where the nucleotide substitutions result in one or more amino acid changes, preferred leptin polypeptides include those that retain the same activities and activity levels as the leptin polypeptide encoded by the reference polynucleotide sequence, as well as those where the level of one or more activities is increased, and alternatively where the level of one or more activities is decreased or even absent. Leptin polypeptide activities of the invention are described herein in the Examples in more detail (1-8, 10 & 14), but include LSR binding leading to the uptake and degradation of leptin, as well as the upregulation of LSR receptors that bind, uptake and degrade triglycerides. Examples of assays to determine the presence or absence of specific leptin activities and the level of the activity(s) are also described herein.

By "retain the same activities" is meant that the activity measured using the polypeptide encoded by the variant leptin polynucleotide in assays is at least 75% (preferably at least 85%, more preferably at least 95%, most preferably at least 98%) and not more than 125% (preferably not more than 115%, more preferably not more than 105%, most preferably not more than 102%) of the activity measured using the leptin polypeptide encoded by the reference sequence.

By the activity being "increased" is meant that the activity measured using the polypeptide encoded by the variant leptin polynucleotide in assays is at least 125% (preferably at least 150%, more preferably at least 200%, most preferably at least 500%) of the activity measured using the leptin polypeptide encoded by the reference sequence.

By the activity being "decreased" is meant that the activity measured using the polypeptide encoded by the variant leptin polynucleotide in assays is not more than 75% (preferably not more than 50%, more preferably not more than 25%, most preferably not more than 10%) of the activity measured using the leptin polypeptide encoded by the reference sequence.

By the activity being "absent" is meant that the activity measured using the polypeptide encoded by the variant leptin polynucleotide in assays is less than 25%, alternatively less than 10% (preferably less than 5%, more preferably less than 2%, most preferably less than 1%) of the activity measured using the leptin polypeptide encoded by the reference sequence.

A polynucleotide fragment is a polynucleotide having a sequence that entirely is the same as part, but not all, of a given nucleotide sequence, preferably the nucleotide sequence encoding a leptin polypeptide that binds and activates LSR, and variants thereof as described above, and the complements of these polynucleotides. Such fragments may be "free-standing", *i.e.* not part of or fused to other polynucleotides, or they may be comprised within a single larger non-leptin polynucleotide of which they form a part or region. However, several fragments may be comprised within a single larger polynucleotide.

Optionally, such fragments may consist of a contiguous span that ranges in length from 8, 10, 12, 15, 18 or 20 to 25, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 nucleotides, or be

specified as being 12, 15, 18, 20, 25, 35, 40, 50, 60, 70 , 80 , 90, 10, 110, 120, 130, 140, or 150 nucleotides in length.

A preferred embodiment of the invention includes isolated, purified, or recombinant polynucleotides consisting of a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 nucleotides encoding a leptin polypeptide of the invention, or the complements thereof, wherein said contiguous span encodes a fragment of leptin that retains the same activities and activity levels as the leptin polypeptide encoded by the reference polynucleotide sequence, or encodes a fragment of leptin where the level of one or more activities is increased, or alternatively where the level of one or more activities is decreased or even absent as described above.

An additional preferred embodiment of the invention includes isolated, purified, or recombinant polynucleotides consisting of a contiguous span of 8 to 50 nucleotides of a leptin polypeptide of the invention, or their variants, or the complements thereof, wherein said contiguous span encodes a fragment of leptin that retains the same activities and activity levels as the leptin polypeptide encoded by the reference polynucleotide sequence, or encodes a fragment of leptin where the level of one or more activities is increased, or alternatively where the level of one or more activities is decreased or even absent as described above. Any of the above-described fragments may be comprised within a larger non-leptin polynucleotide fragment.

II. Leptin Polypeptide Fragments of the Invention

Leptin polypeptide fragments that bind/activate/modulate LSR have been identified (Examples 1-8). This region was identified by a comparison of the human and murine leptin amino acid sequences, and its activity confirmed *in vitro* and *in vivo* (See Examples 1-8). The advantages to having identified a leptin fragment responsible for leptin activity, include its use (1) as part of an assay system to discover leptin receptors and binding partners (in association with LSR for example), (2) as a lead molecule for the design of other compounds able to modulate LSR activity, and (3) as part of a treatment and/or prevention for obesity-related diseases and disorders. Knowledge of specific polypeptides involved is especially useful since it allows its use in assay systems (rather than the entire protein) and keeps the cost down (easily synthesized). In addition, a peptide can be expected to easily crystallize in the correct conformation to allow structure-function studies to design other small molecule activators. Finally, use of just the active portion in treatment should increase the chances of the peptide remaining active and potentially decreasing side-effects.

Furthermore, in the process of identifying the "active" portion of human leptin for human cells, a corresponding inhibitory portion of mouse leptin for human cells was identified. Comparisons between the two highly similar fragments will enable the identification of important residues for both increasing the activity of LSR and inhibiting the activity of LSR. This will be useful both in competitive assays for

inhibitors and activators of LSR, and also for treatments in mammals and animals where inhibition of LSR is desired.

The invention relates to leptin polypeptides as well as to variants, fragments, analogs and derivatives of the leptin polypeptides described herein, including modified leptin polypeptides. Preferred embodiments of the invention feature a leptin polypeptide that consists of a sequence described in Example 10, or variants, fragments, analogs, or derivatives thereof. Preferably the polypeptides are, purified, isolated and/or recombinant.

In other preferred embodiments, the invention features a leptin polypeptide fragment that modulates the activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of any one of the leptin polypeptide sequences set forth in Figure 13, wherein said at least 4 and not more than 50 contiguous amino acids comprise the leptin fragment central sequence. By the « leptin fragment central sequence » as used herein is meant the four variable amino acids of the active leptin peptide identified in Example 10 by sequence comparisons and molecular modeling. These residues comprise ETLD (SEQ ID NO:40) and QKPE (SEQ ID NO:41) for the human and mouse sequences, respectively, in Fig. 13. Preferably, the leptin polypeptide fragment comprises at least 10, but not more than 50, more preferably at least 15 but not more than 40, or at least 20 and not more than 40, or most prefereably at least at least 15 but not more than 30, or 20 but not more than 30 contiguous amino acids of any one of the leptin polypeptide sequences set forth in Figure 13, wherein said contiguous amino acids comprise the leptin polypeptide variable region. Preferably the leptin polypeptide fragment is human or mouse, but most preferably human, or a derivative or variant thereof.

Variant leptin polypeptides of the invention may be 1) ones in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, or 2) ones in which one or more of the amino acid residues includes a substituent group, or 3) ones in which a modified leptin polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or 4) ones in which the additional amino acids are fused to a modified leptin polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the modified leptin polypeptide or a pre-protein sequence. Such variants are deemed to be within the scope of those skilled in the art.

Amino acid changes present in a variant polypeptide may be non-conservative amino acid changes but more preferably are conservative amino acid changes. In cases where there are one or more amino acid changes, preferred leptin polypeptides include those that retain the same activities and activity levels as the reference leptin polypeptide sequence, as well as those where the level of one or more activities is increased, and alternatively where the level of one or more activities is decreased or even absent. Assays for determining leptin polypeptide activities of the invention are described herein in the Examples (1-8 & 13) in more detail, but include LSR binding leading to the uptake and degradation

of leptin, as well as the upregulation of LSR receptors that bind, uptake and degrade triglyceride-rich lipoproteins. Examples of assays to determine the presence or absence of specific leptin activities and the level of the activity(s) are also described herein. Definitions of activities are provided in "Leptin Polynucleotides of the Invention" (section I).

In preferred embodiments, the invention features a variant of a leptin polypeptide fragment that modulates the activity of LSR, consisting of a 22 contiguous amino acid sequence that is at least 75% identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13. By the « leptin fragment variable region » as used herein is meant the region of 22 amino acids that is shaded in figure 13 for all the species in the alignment. Preferably, the 22 contiguous amino acid sequence is at least 85% identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13, more preferably 90% identical, most preferably 95% identical and optionally 100% identical. Preferably the sequence is human or mouse, and most preferably human.

In yet other preferred embodiments, the invention features a variant of a leptin polypeptide fragment that modulates the activity of LSR, consisting of a 22 contiguous amino acid sequence, wherein at least 16 of the 22 amino acids are identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13. Preferably, at least 18 of the 22 amino acids are identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13, more preferably 20 of the 22 are identical, most preferably all of the amino acids are identical. Preferably the sequence is human or mouse, and most preferably human.

A polypeptide fragment is a polypeptide having a sequence that is entirely the same as part, but not all, of a given polypeptide sequence, preferably a polypeptide encoded by a leptin gene and variants thereof. Such fragments may be "free-standing", *i.e.* not part of or fused to other polypeptides, or they may be comprised within a single larger non-leptin polypeptide of which they form a part or region. However, several fragments may be comprised within a single larger polypeptide. As representative examples of polypeptide fragments of the invention, there may be mentioned those which have from about 4, 5, 6, 7, 8, 9 or 10 to 15, 10 to 20, 15 to 40, or 30 to 55 amino acids long. Preferred are those fragments containing at least one amino acid substitution or deletion in a leptin polypeptide.

The present invention is particularly focused on a set of variant leptin polypeptides and the fragments thereof. A preferred set of polypeptides of the invention include isolated, purified, or recombinant polypeptides comprising a contiguous span of at least 3 (preferably at least 6, more preferably at least 10, most preferably at least 15) amino acids of any of the leptin fragment variable regions of the sequences provided in Figure 13.

III. Zinc Finger Proteins of the Invention

Zinc finger proteins of the Cys2His2 type are malleable DNA binding proteins that can be designed to bind diverse sequences, and that typically contain 3 zinc finger domains. The inventors contemplate the use of any zinc finger protein engineered to bind the DNA of interest, specifically. Although six-fingered proteins have been described to target unique sites within the genome (International Publication WO 98/54311, hereby incorporated herein by reference in its entirety including any figures, tables and drawings) proteins with different numbers of fingers that are engineered to bind specifically to the genome are also included in the invention. The six-fingered proteins described in WO 98/54311, bind two 9 contiguous base pair fragments (separated by 0, 1, 2, or 3 nucleotides) of DNA or RNA in a sequence specific fashion, and can be used to regulate gene transcription. The zinc finger proteins of the invention also include those that are designed to bind sequences a greater distance apart and thereby confer greater specificity with fewer (or the same number, or more) "fingers". Methods for designing the zinc finger proteins of the invention, as well as for determining the sequences to which the zinc finger proteins bind, are described in International Publication WO 98/54311 entitled "Zinc Finger Protein Derivatives and Methods Therefor".

For one embodiment of the invention, zinc finger proteins have been designed that will bind to the 5' regulatory regions and selected introns of LSR and thereby inhibit or augment the transcription of endogenous LSR as described herein (Example 12). Exogenous LSR that is introduced into the cell without these regulatory regions or introns (cDNA) will be expressed normally. This can be useful *in vitro* both as a research tool to study the role of the various LSR components in leptin signaling and triglyceride-rich lipoprotein uptake and degradation, for example, and as part of an assay to discover modulators of LSRlep and LSRtg activity. Therefore, in currently preferred embodiments, zinc finger proteins are not designed to bind to the exons of LSR. However, in circumstances where no endogenous nor exogenously-introduced LSR activity is desired in a cell, for example, zinc finger proteins designed to bind to LSR exons could be useful.

The invention features a zinc finger protein, comprising a DNA binding domain that binds specifically to 18 nucleotides of a sequence at least 50% homologous to SEQ ID NO:1, wherein said 18 nucleotides comprise two fragments of 9 contiguous nucleotides, and wherein said fragments are separated by 0, 1, 2, or 3 nucleotides. In preferred embodiments, the zinc finger protein binds to sequences that are at least 50% homologous to the sequence of the introns of SEQ ID NO:1. Preferably, the sequence is at least 50% homologous to the sequence of the first intron of SEQ ID NO:1. In other preferred embodiments, the zinc finger protein binds specifically to 18 nucleotides of a sequence that is 75% identical, 80%, 85%, or 90% identical, or most preferably 99 to 100% identical to SEQ ID NO :1, the introns of SEQ ID NO :1, or preferably the first intron of SEQ ID NO :1.

In preferred embodiments of the invention, the zinc finger protein of the invention further comprises a functional domain selected from the group consisting of a transcription repressor and a transcription initiator. These repressors and initiators can be any that are known in the art. Preferably, the repressor is a KRAB

repressor and the initiator is a VP16 initiator. In highly preferred embodiments, the protein further comprises a small molecule regulatory system that can be any known in the art; however, the system is preferably selected from the group consisting of a Tet system, RU486, and ecdysone.

It is envisioned that zinc finger proteins could be designed to bind to any 18 or more contiguous base pairs of a sequence at least 50%, preferably 75%, more preferably 90%, most preferably 95% identical to the 5' regulatory region (for example, residues 1-2000 of SEQ ID NO:1) or any of the introns of LSR (for example, 2357 to 3539, 3885 to 12162, 12283 to 15143, 15201 to 17764, 15912 to 19578, 19753 to 19898, 19959 to 20055, 20188 to 20328, and 20958 to 21046 of SEQ ID NO:1), and more preferably residues 2357 to 3539 of SEQ ID NO:1. In particular, introns within 3,000 base pairs of the LSR start site are preferred, for example introns 1 through 3.

Guidance is available for determining optimal base pair stretches for zinc finger protein binding, and for determining what zinc finger amino acids will bind to what DNA sequences (WO 98/54311). This information has been used to design an algorithm for designing zinc finger proteins available from Sangamo BioSciences. However, as described in WO 98/54311, zinc finger proteins for binding a given piece of DNA can be identified by screening or "panning" libraries of zinc finger proteins with the DNA sequence. Zinc finger libraries can be made, for example, by randomly mutating genes encoding known zinc finger proteins (WO 98/54311). The effectiveness of the zinc finger protein identified by the panning procedure can then be assessed in the *E. coli* method described in WO 98/54311 (co-transfection of genes encoding the zinc finger protein and the gene of which the DNA sequence makes up a part). The effectiveness of the zinc finger protein for inhibiting LSR expression can be further tested using the assay systems described in the Examples (1-8); in particular the use of FACS following staining with an LSR specific antibody and quantitative PCR will be useful.

In preferred embodiments, addition of the zinc finger protein preferably inhibits LSR transcription completely, or inhibits LSR translation completely. By "inhibits transcription completely" is meant that the level of transcription following addition of the zinc finger protein is preferably below the level of detection by the assay used as compared to control cells. The assay used may be a Northern blot, or any other assay that measures RNA expression, such as quantitative PCR. Alternatively, the level of transcription of LSR may be significantly reduced. By "significantly reduced" is meant that the amount of RNA is preferably reduced at least 2-fold, more preferably at least 5-fold, and most preferably at least 10-fold compared to the level RNA prior to the addition of the zinc finger protein, or the level in control cells.

Similarly, by "inhibits translation completely" is meant that LSR protein is preferably below the level of detection by the assay used compared with control cells. The assay used may be a Western blot, or dot blot, or other type of immunoassay for example, or any other assay known in the art to be used to measure or detect the presence of proteins, such as FACS with fluorescent antibodies to LSR. Alternatively, the level of translation of LSR may be significantly reduced. By "significantly reduced"

is meant the amount of protein present is preferably reduced at least 2-fold, more preferably at least 5-fold, most preferably at least 10-fold compared to the level of protein present prior to the addition of zinc finger protein, or in control cells.

Highly preferred sequences to be used for designing zinc finger proteins include, residues 1841 to 1860, 1880 to 1898, 1918 to 1945, 1951 to 1973, and 3362 to 3382 of human LSR (SEQ ID NO:1) and of the homologous regions in genes coding for LSR proteins of other species, preferably including mouse and rat LSR. The genomic sequences encoding LSR from other species can be identified by methods well-known in the art.

These zinc finger proteins can also be useful *in vivo* both as part of an assay system in animal models to discover modulators of LSRlep (at least α', may include β and/or α) and LSRtg (at least α, may include β and/or α') activity, as well as in gene surgery in which transcription of endogenous LSR is inhibited as part of the treatment for an obesity-related disease or disorder. This could be useful in a case where the LSR message was being over-expressed, or incorrectly expressed (mutated), for example. A potential therapy would include providing this zinc finger protein alone, in cases of simple over-expression, or in conjunction with other appropriate components of LSR if the cellular LSR was mutated. These proteins could be targeted to the appropriate cells (those with LSR) by using liposomes, for example, with leptin or another LSR binding protein in the liposome membrane.

In an alternative embodiment of the invention, zinc finger proteins are designed to bind to the 5' regulatory regions of LSR and thereby increase the transcription of endogenous LSR. Typically, within the 5' regulatory region of genes are promoters as well as other regulatory elements. Binding of zinc finger proteins to certain regions of the DNA may serve to facilitate binding of the initiation complex and thus transcription of the gene. For instance, where some unusual folding prevents access to the promoter region, if a zinc finger protein were to bind the DNA upstream such that the folding were prevented, then the promoter would have greater access and enhanced transcription should result. Alternatively, it may be possible to design a zinc finger protein that binds the promoter region directly, thereby initiating transcription.

In these and other circumstances, zinc finger binding proteins designed to bind stretches of DNA in the 5'regulatory region as described above can be tested for their ability to enhance transcription of LSR. Thus, in preferred embodiments, addition of the zinc finger protein preferably significantly increases LSR transcription, or significantly increases LSR translation. By "significantly increases LSR transcription" is meant that the level of transcription following addition of the zinc finger protein is preferably increased at least 2-fold, more preferably at least 5-fold, and most preferably at least 10-fold compared to the level RNA prior to the addition of the zinc finger protein. The assay used may be a Northern blot, or any other assay that measures RNA expression. Alternatively, if the starting level of RNA transcription is below the level of detection by the assay used, "significantly increases LSR

transcription" may mean that the level of transcription of LSR may become detectable on the addition of the zinc finger binding protein.

Similarly, by "significantly increases LSR translation" is meant that the level of translation following addition of the zinc finger protein is preferably increased at least 2-fold, more preferably at least 5-fold, and most preferably at least 10-fold compared to the level of translation prior to the addition of the zinc finger protein. The assay used may be a Western blot, or dot blot, or other type of immunoassay for example, or any other assay known in the art to be used to measure or detect the presence of proteins. Alternatively, if the starting level of LSR protein is below the level of detection by the assay used, "significantly increases LSR translation" may mean that LSR protein may become detectable after the addition of the zinc finger binding protein.

These zinc finger proteins can be useful *in vivo* in gene surgery in which transcription of endogenous LSR is enhanced as part of the treatment for an obesity-related disease or disorder. This can be envisioned in a situation where higher levels of the LSR protein are thought to be advantageous for the patient clinically. For example, increased expression of LSR could be advantageous when the LSR gene is normal, but is expressed at lower than normal levels, or when it is expressed at normal levels, but does not function as efficiently as it should in clearing triglycerides from the bloodstream, or when some other abnormality results in abnormally high levels of triglycerides and an increased amount of LSR protein is necessary to clear them.

In a further alternative embodiment of the invention, zinc finger proteins are designed to bind to any sequence of 18 or more contiguous base pairs of LSR mRNA and thereby inhibit translation of LSR. In preferred embodiments, expression of all three forms of LSR are inhibited by the zinc finger protein. In an alternative embodiment, zinc finger proteins are designed to specifically inhibit expression of the LSR α, α', or β subunit individually, or to inhibit both the LSR α and α' subunits. All three forms of LSR can be inhibited by zinc finger proteins targeted to mRNA fragments transcribed from exons one through 3 and exon 6 to the end. The α subunit can be targeted with zinc finger proteins designed to bind in exon 4. The α' subunit can be targeted with zinc finger proteins designed to bind to the splice site between exon 3 and exon 5. The β subunit can be targeted with zinc finger proteins designed to bind to the splice site between exon 3 and exon 6. Both the α and α' subunits can be targeted with zinc finger proteins designed to bind to exon 5.

These zinc finger proteins would be useful for many of the uses previously described for zinc finger proteins binding to and inhibiting or increasing transcription of LSR DNA. Similarly the definitions for inhibiting or increasing LSR transcription and tests for the desired zinc finger proteins and methods for designing and making them would be as previously described. In addition, for all of the zinc fingers described, it should be remembered that the system can be further controlled by addition of a small molecule control system (for example the Tet-responsive system, or RU486, or ecdysone) to the cell. This allows greater control/greater finesse for an *in vitro* assay system, in particular, but can be

used *in vivo* as well. The basic idea is to provide the zinc finger with part of the Tet system integrated upstream such that transcription of the zinc finger protein can be regulated by the addition of an outside element, for example Dox or Tc. These methods are well-known to those in the art.

IV. Polynucleotides Encoding Zinc Finger Polypeptides of the Invention

The invention also features polynucleotides that encode the zinc finger polypeptides of the invention described above. In one method of identifying the desired zinc finger polypeptides of the invention, libraries are screened (panned) for those clones expressing a zinc finger protein that binds to the desired nucleotide sequence. Frequently, multiple clones are identified that express zinc finger proteins that bind to the nucleotide sequence. All the variant polynucleotides that code for the zinc finger polypeptide(s) that bind to the desired sequence are also part of the present invention.

Variants of polynucleotides, as the term is used here, are polynucleotides whose sequence differs from a reference polynucleotide; in this case a reference polynucleotide is the polynucleotide that is ultimately chosen to be used. Thus, the variant of the polynucleotide would frequently be the result of mutagenesis techniques as described in WO 98/54311. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical.

Nucleotide changes present in a variant polynucleotide are preferably silent, which means that they do not alter the amino acids encoded by the polynucleotide. However, nucleotide changes may also result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. The substitutions, deletions or additions may involve one or more nucleotides. Alterations in the zinc finger polypeptide coding regions of the invention may produce conservative or non-conservative amino acid substitutions, deletions or additions in the encoded protein. Preferably, the nucleotide substitutions result in non-conservative amino acid changes and more preferably in conservative amino acid changes in the encoded polypeptide.

In cases where the nucleotide substitutions result in one or more amino acid changes, preferred zinc finger polypeptides include those that retain the same activities and activity levels as the zinc finger polypeptide encoded by the reference polynucleotide sequence, as well as those where the level of one or more activities is increased, and alternatively where the level of one or more activities is decreased or even absent. Zinc finger polypeptide activities of the invention and methods for testing are described above.

A polynucleotide fragment is a polynucleotide having a sequence that entirely is the same as part, but not all, of a given nucleotide sequence, preferably the nucleotide sequence encoding a zinc finger polypeptide, and variants thereof, as described above, and the complements of these polynucleotides. Such fragments may be "free-standing", *i.e.* not part of or fused to other polynucleotides, or they may be comprised within a single larger polynucleotide of which they form a part or region. However, several

fragments may be comprised within a single larger polynucleotide. Optionally, such fragments may consist of a contiguous span that ranges in length from 8, 10, 12, 15, 18 or 20 to 25, 35, 40, 50, or 60 nucleotides, or be specified as being 12, 15, 18, 20, 25, 35, 40, 50, or 60 nucleotides in length.

V. Chimeric Oligonucleotides of the Invention

Chimeraplasty is a technique used to change the nucleotide sequence of DNA of cells and of animals (Science 285 :316-318 (1999)). It can be used to create or to correct mutations, usually point mutations, that have an effect on the protein coding sequence. The technique relies on hybrid molecules of DNA and RNA called chimeras that contain DNA with a mutation in its sequence (compared to the target sequence in the cell) flanked by RNA that perfectly mirrors the flanking target gene sequence. The target gene sequence is thought to be modified through the action of the cell's DNA repair machinery as a result of the pairing of the target DNA with the chimera containing the mutated sequence.

In the present invention, the advantages to using chimeraplasty to modify LSR include : (1) ease of creating cells lacking LSR polypeptides for use in assays or gene surgery; (2) specifically blocking production of the α subunit or the α and α' subunits for use in assays or in gene surgery; and (3) the ability to correct defects in the LSR gene in cells *in vitro* and *in vivo* for use in gene surgery. Chimeraplasty has been shown to be effective for correcting (or creating) mutations in cells *in vitro* and *in vivo* in animals (Cole-Strauss, et al. Science 273 : 13861389 (1996) ; Alexeev and Yoon Nature Biotechnology 16 : 1343-1346 (1998) ; Kren et al Nature Medecine 4 : 285-290 (1998) ; Yoon et al Proc Natl. Acad. Sci. USA 93 : 2071-2076 (1996) ; Xiang et al J Mol Med 75 : 829-825 (1997), hereby encorporated by reference herein in their entirety including any figures, drawings, or tables). Chimeraplasty is particularly useful in cases of point mutations that need to be corrected to allow either expression or function of the protein.

Chimeraplasty apparently works through the cell's own DNA repair system to correct the targeted gene. Although the gene is not corrected in 100% of the cells following transfection *in vitro* or introduction into the animal *in vivo*, the genes in enough of the cells have been found to be changed to permit a clinically detectable change. This could, in fact, be beneficial in the LSR system where it is unlikely that you would ever want to completely prevent LSR expression. However, reduction in LSR expression might be advantageous in some obesity-related diseases and disorders. In particular, specific reduction in any one or more of the α, α', or β subunits could be advantageous.

The invention features a chimeric oligonucleotide, comprising at least 9 contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16, wherein said at least 9 contiguous nucleotides comprise at least one amino acid codon selected from the group consisting of TTA, TTG, TCA, TCG, TAU, TAC, TGT, TGC, TGG, CAA, CAG, AGA, GAA, GAG, and GGA, and wherein a point mutation is present in said codon such that said codon is a stop codon. In preferred embodiments, the sequence

is selected from the group consisting of Exon 1, Exon 4 and Exon 5 from SEQ ID NO : 1 and homologous sequences from mouse or rat, preferably mouse.

Another embodiment of the invention features chimeraplast LSR polynucleotides, where the polynucleotide comprises at least 7 (preferably at least 13, more preferably at least 25, most preferably at least 35 nucleotides of the LSR gene (or its complement), and where the DNA portion of the chimera comprises a point mutation such that instead of coding for an amino acid, it now codes for a termination codon. Thus, substitutiton of this nucleotide for the nucleotide present in the endogenous LSR gene, results in a stop codon being created at the site. The other nucleotides present in both the DNA and RNA portions of the chimera are 100% complementary to the flanking regions of the endogenous LSR gene. The DNA portion of the chimera is at least 3 consecutive nucleotides in length, preferably at least 5 consecutive nucleotides in length, optionally at least 7 or at least 11 nucleotides in length. The point mutation is preferably the middle nucleotide (n ; alternatively n+1, or n-1 ; less preferably n+2, or n-2 ; n+3, or n-3, etc.) of the DNA part of the chimera when the DNA portion has an odd number of nucleotides (AGnCT, AnGCT, AGCnT, for example), or the n+1 or n-1 positions (less preferably n+2, or n-2 ; n+3, or n-3, etc.) when the sequence has an even number of nucleotides (AnCT, AcnT, for example). The RNA portion of the chimera is at least 4 consecutive nucleotides in length, preferably at least 10 consecutive nucleotides in length, more preferably at least 20 consecutive nucleotides in length, and most preferably at least 30 consecutive nucleotides in length. The RNA portion of the chimera flanks the DNA portion of the chimera, preferably with an equal number of nucleotides on each side of the DNA sequence (x ; when the number on RNA residues is even), less preferably with x+1 on the upstream side and x-1 on the downstream side or alternatively x+1 on the downstream side and x-1 on the upstream side ; even less preferably with x+2 on the upstream side and x-2 on the downstream side or alternatively x+2 on the downstream side and x-2 on the upstream side, and so on. Similarly, when the number of RNA residues is odd, there are either x+1 on the upstream side and x-1 on the downstream side or alternatively x+1 on the downstream side and x-1 on the upstream side of the DNA; less preferably there are x+2 on the upstream side and x-2 on the downstream side or alternatively x+2 on the downstream side and x-2 on the upstream side, and so on. In some cases, particularly when the point mutation is not in the center of the DNA part of the chimera, the number of residues of RNA flanking the DNA is preferably not equal on both sides. In some cases it is preferred that there are more RNA residues on one side than the other so as to have the point mutation be located at the center of the chimera, or at least n+1 or n-1 from the center of the chimera, less preferably n+2, or n-2 from the center, etc. Sequences that encode stop codons include TAA, TAG, and TGA. Therefore, sequences encoding the amino acids leucine (TTA or TTG), serine (TCA or TCG), tyrosine (TAU or TAC), cysteine (TGT or TGC), tryptophan (TGG), glutamine (CAA or CAG), arginine (AGA), glutamate (GAA or GAG), or glycine (GGA), for example, can be changed to one of the stop codons by a single polynucleotide exchange. The preferred stop codon is TGA. The exact design of the chimeras will depend on the

particular sequence to be mutated, but guidance has been given in the papers listed above and in the Examples herein. In general, however, the sequence should be at least 14 nucleotides in length (preferably 18, more preferably 25, most preferably 30) to ensure specificity to the desired sequence. Preferably, the amino acid to be mutated to a termination codon is located at the 5' end of the coding sequence, preferably within the first exon, and preferably is the first amino acid that can be mutated in this way after the first ATG or most preferably the second ATG. Amino acids to be mutated to stop all LSR expression should not be selected from Exon 4 or Exon 5, since exon 4 is not present in the α' subunit, and neither Exon 4 nor Exon 5 is present in the β subunit. The success of a chimeraplast in preventing LSR expression can be tested using the techniques described herein, to include screens for the presence of the mRNA by Northern blot, for example, and for the protein by Western blot, for example.

Alternatively, in some preferred embodiments it is preferable to stop expression of the LSR α subunit only. To do this, the amino acid to be mutated is preferably located in Exon 4 of LSR, since this Exon is not present in the α' or β subunits. In other preferred embodiments it is preferable to prevent expression of both α and α' subunits, but not the β subunit. To do this, the amino acid to be mutated is preferably located in Exon 5 of LSR, since this exon is present in both α and α' subunits, but not the β subunit.

In another embodiment, the invention feaures chimeraplast LSR polynucleotides, where the polynucleotide comprises at least 7 (preferably at least 13, more preferably at least 25, most preferably at least 35 nucleotides of the LSR gene (or its complement), and where the DNA portion of the chimera comprises one of the alleles of the single nucleotide polymorphisms (SNPs) described in U.S. Provisional Application No. 60/119, 592, entitled « Polymorphic Markers of the LSR Gene » by Blumenfeld, Bougueleret, and Bihain, filed February 10, 1999 and previously incorporated by reference herein, and indicated in Table A. Preferably, the SNP's are selected from the group consisting of A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A23, A24, A25, A26, A27, A28, A29, A30, A31, AND A32. The SNPs may be in either coding or non-coding regions of the LSR gene. Some SNPs in the coding region result in amino acid changes that may affect the activity of LSR. However, the majority of the SNPs do not code for amino acid changes. These nucleotide changes can also modulate the activity of LSR in a variety of ways, for example by interfering with the binding of a regulatory molecule that influences the splicing of the introns, particularly where there is differential splicing depending on the subunit to be expressed or by affecting the binding of promoters or the function of other regulatory sequences in the 5' and 3' regions of the gene. Changes in the expression of various subunits, or the levels of expression of LSR in general, can have profound effects on the obesity of patients.

VI. Recombinant Vectors of the Invention

The term "vector" is used herein to designate either a circular or a linear DNA or RNA molecule, that is either double-stranded or single-stranded, and that comprises at least one polynucleotide of interest that is sought to be transferred in a cell host or in a unicellular or multicellular host organism.

The present invention relates to recombinant vectors comprising any one of the polynucleotides described herein.

The present invention encompasses a family of recombinant vectors that comprise polynucleotides encoding leptin polypeptides of the invention, polynucleotides encoding zinc finger proteins of the invention, and chimeraplastic polynucleotides of the invention as described herein.

In a first preferred embodiment, a recombinant vector of the invention is used to amplify the inserted polynucleotide in a suitable cell host, this polynucleotide being amplified every time that the recombinant vector replicates. The inserted polynucleotide can be one that encodes leptin polypeptides of the invention or zinc finger polypeptides of the invention, or a chimeraplast polynucleotide.

A second preferred embodiment of the recombinant vectors according to the invention, consists of expression vectors comprising either a polynucleotide encoding leptin polypeptides of the invention or zinc finger proteins of the invention, or both. Within certain embodiments, expression vectors are employed to express a leptin polypeptide of the invention, preferably a modified leptin polypeptide described in the present invention, which can be then purified and, for example, be used in screening assays or as a treatment for obesity-related diseases. In other embodiments, expression vectors are employed to express a zinc finger protein of the invention, preferably one that inhibits LSR expression or expression of specific subunits of LSR as described in the present invention, which can be then purified and, for example, be used in screening assays or as a treatment for obesity-related diseases. In other embodiments, the expression vectors are used for constructing transgenic animals and also for gene surgery, in particular, expression vectors containing a polynucleotide encoding zinc finger proteins of the invention.

Expression requires that appropriate signals are provided in the vectors, said signals including various regulatory elements, such as enhancers/promoters from both viral and mammalian sources, that drive expression of the genes of interest in host cells. Dominant drug selection markers for establishing permanent, stable, cell clones expressing the products are generally included in the expression vectors of the invention, as they are elements that link expression of the drug selection markers to expression of the polypeptide.

More particularly, the present invention relates to expression vectors which include nucleic acids encoding a leptin polypeptide fragment of the invention, or a modified leptin polypeptide as described herein, or variants or fragments thereof, under the control of a regulatory sequence selected among the leptin regulatory polynucleotides, or alternatively under the control of an exogenous regulatory sequence. The present also relates to expression vectors which include nucleic acids encoding a zinc finger

polypeptide of the invention, or a modified zinc finger polypeptide as described herein, or variants or fragments thereof, under the control of an exogenous regulatory sequence.

Consequently, preferred expression vectors of the invention are selected from the group consisting of : (a) a leptin regulatory sequence and driving the expression of a coding polynucleotide operably linked thereto; (b) a leptin polypeptide coding sequence of the invention, operably linked to regulatory sequences allowing its expression in a suitable cell host and/or host organism. Other preferred expression vectors of the invention comprise a zinc finger polypeptide coding sequence of the invention, operably linked to regulatory sequences allowing its expression in a suitable cell host and/or host organism.

Some of the elements which can be found in the vectors of the present invention are described in further detail in the following sections.

1) General features of the expression vectors of the invention :

A recombinant vector according to the invention comprises, but is not limited to, a YAC (Yeast Artificial Chromosome), a BAC (Bacterial Artificial Chromosome), a phage, a phagemid, a cosmid, a plasmid, or even a linear DNA molecule which may consist of a chromosomal, non-chromosomal, semi-synthetic or synthetic DNA. Such a recombinant vector can comprise a transcriptional unit comprising an assembly of :

(1) a genetic element or elements having a regulatory role in gene expression, for example promoters or enhancers. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp in length that act on the promoter to increase the transcription.

(2) a structural or coding sequence which is transcribed into mRNA and eventually translated into a polypeptide, said structural or coding sequence being operably linked to the regulatory elements described in (1); and

(3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, when a recombinant protein is expressed without a leader or transport sequence, it may include a N-terminal residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

Generally, recombinant expression vectors will include origins of replication, selectable markers permitting transformation of the host cell, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably a leader sequence capable of directing secretion of the translated protein into the periplasmic space or the extracellular medium. In a specific embodiment wherein the vector is adapted for transfecting and expressing desired sequences in mammalian host cells, preferred vectors will comprise an origin of replication in the desired

host, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking non-transcribed sequences. DNA sequences derived from the SV40 viral genome, for example SV40 origin, early promoter, enhancer, splice and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

2) Regulatory elements

Promoters

The suitable promoter regions used in the expression vectors according to the present invention are chosen taking into account the cell host in which the heterologous gene has to be expressed. The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell, such as, for example, a human or a viral promoter.

A suitable promoter may be heterologous with respect to the nucleic acid for which it controls the expression or alternatively can be endogenous to the native polynucleotide containing the coding sequence to be expressed. Additionally, the promoter is generally heterologous with respect to the recombinant vector sequences within which the construct promoter/coding sequence has been inserted.

Promoter regions can be selected from any desired gene using, for example, CAT (chloramphenicol transferase) vectors and more preferably pKK232-8 and pCM7 vectors. Preferred bacterial promoters are the LacI, LacZ, the T3 or T7 bacteriophage RNA polymerase promoters, the gpt, lambda PR, PL and trp promoters (EP 0036776), the polyhedrin promoter, or the p10 protein promoter from baculovirus (Kit Novagen) (Smith et al. (1983) *Mol. Cell. Biol.* 3:2156-2165.; O'Reilly et al., 1992, Baculovirus expression vectors : a Laboratory Manual. W.H. Freeman and Co., New York the lambda PR promoter or also the trc promoter.

Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-L. Selection of a convenient vector and promoter is well within the level of ordinary skill in the art.

The choice of a promoter is well within the ability of a person skilled in the field of genetic egineering. For example, one may refer to the book of (Sambrook, J., Fritsch, E.F., and T. Maniatis. (1989), *Molecular Cloning: A Laboratory Manual.* 2ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York))or also to the procedures described by (Fuller S. A. et al. (1996) *Immunology in Current Protocols in Molecular Biology*, Ausubel et al., Eds, John Wiley & Sons, Inc., USA).

Other regulatory elements

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

The vector containing the appropriate DNA sequence as described above, more preferably *LSR* gene inhibitory or activating polynucleotide, a polynucleotide encoding a leptin polypeptide or both of them, can be utilized to transform an appropriate host to allow the expression of the desired polypeptide or polynucleotide.

3) Selectable markers

Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. The selectable marker genes for selection of transformed host cells are preferably dihydrofolate reductase or zeocin, hygromycin or neomycin resistance for eukaryotic cell culture, TRP1 for *S. cerevisiae* or tetracycline, rifampicin or ampicillin resistance in *E. coli*, or levan saccharase for mycobacteria, this latter marker being a negative selection marker.

4) Preferred vectors

Bacterial vectors

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and a bacterial origin of replication derived from commercially available plasmids comprising genetic elements of pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia, Uppsala, Sweden), and GEM1 (Promega Biotec, Madison, WI, USA).

Large numbers of other suitable vectors are known to those of skill in the art, and are commercially available, such as the following bacterial vectors : pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pMSG, pSVL (Pharmacia); pQE-30 (QIAexpress).

Baculovirus vectors

A suitable vector for the expression polypeptides of the invention is a baculovirus vector that can be propagated in insect cells and in insect cell lines. A specific suitable host vector system is the pVL1392/1393 baculovirus transfer vector (Pharmingen) that is used to transfect the SF9 cell line (ATCC N°CRL 1711) which is derived from *Spodoptera frugiperda*.

Other suitable vectors for the expression of a leptin polypeptide in a baculovirus expression system include those described by (Chai H. et al. (1993), *Biotechnol. Appl. Biochem.*18:259-273; Vlasak R. et al. (1983), *Eur. J. Biochem.* 135:123-126; Lenhard T. et al. (1996), *Gene.* 169:187-190).

Viral vectors

In one specific embodiment, the vector is derived from an adenovirus. Preferred adenovirus vectors according to the invention are those described by Feldman and Steg (1996) or Ohno et al. (1994). Another preferred recombinant adenovirus according to this specific embodiment of the present invention is the human adenovirus type 2 or 5 (Ad 2 or Ad 5) or an adenovirus of animal origin (French patent application N° FR-93.05954).

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery systems of choice for the transfer of exogenous polynucleotides *in vivo* , particularly to mammals, including humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. Particularly preferred retroviruses for the preparation or construction of retroviral *in vitro* or *in vitro* gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Mink-Cell Focus Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma virus. Particularly preferred Murine Leukemia Viruses include the 4070A and the 1504A viruses, Abelson (ATCC No VR-999), Friend (ATCC No VR-245), Gross (ATCC No VR-590), Rauscher (ATCC No VR-998) and Moloney Murine Leukemia Virus (ATCC No VR-190; PCT Application No WO 94/24298). Particularly preferred Rous Sarcoma Viruses include Bryan high titer (ATCC Nos VR-334, VR-657, VR-726, VR-659 and VR-728). Other preferred retroviral vectors are those described in Roth J.A. et al. (1996), *Nature Medicine.* 2(9):985-991 PCT Application No WO 93/25234, PCT Application No WO 94/ 06920, Roux et al., 1989, *Proc. Natl Acad. Sci. USA*, 86 : 9079 – 9083, Julan et al., 1992, *J. Gen. Virol.*, 73 : 3251 – 3255 Neda et al., 1991, *J. Biol. Chem.*, 266 : 14143 – 14146.

Yet another viral vector system that is contemplated by the invention consists of the adeno-associated virus (AAV). The adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka et al., 1992, *Curr. Topics in Micro. and Immunol.*, 158 : 97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (Flotte et al., 1992, *Am. J. Respir. Cell Mol. Biol.*, 7:349-356; Samulski et al., 1989, *J. Virol.*, 63 : 3822-3828;

McLaughlin B.A. et al. (1996), *Am. J. Hum. Genet.* 59:561-569. One advantageous feature of AAV derives from its reduced efficacy for transducing primary cells relative to transformed cells.

5) Delivery of the recombinant vectors

In order to effect expression of the polynucleotides of the invention, these constructs must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cell lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states.

One mechanism is viral infection where the expression construct is encapsulated in an infectious viral particle.

Several non-viral methods for the transfer of polynucleotides into cultured mammalian cells are also contemplated by the present invention, and include, without being limited to, calcium phosphate precipitation (Graham et al. (1973), *Virology.* 52:456-457; Chen et al., 1987, Mol. Cell. Biol., 7 : 2745-2752;), DEAE-dextran (Gopal, 1985, Mol. Cell. Biol., 5 : 1188-1190 electroporation (Tur-Kaspa et al. (1986), *Mol. Cell.* Biol. 6:716-718; Potter et al., 1984, Proc Natl Acad Sci U S A. 81(22):7161-5) direct microinjection (Harland et al., 1985, J. Cell. Biol., 101:1094-1095) DNA-loaded liposomes (Nicolau et al., 1982, Biochim. Biophys. Acta, 721:185-190; Fraley et al., 1979, Proc. Natl. Acad. Sci. USA, 76 : 3348-3352 and receptor-mediate transfection (Wu and Wu, 1987, J. Biol. Chem, 262 : 4429-4432; Wu and Wu,1988, Biochemistry, 27:887-892). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression polynucleotide has been delivered into the cell, it may be stably integrated into the genome of the recipient cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle.

One specific embodiment for a method for delivering a protein or peptide to the interior of a cell of a vertebrate *in vivo* comprises the step of introducing a preparation comprising a physiologically acceptable carrier and a naked polynucleotide operatively coding for the polypeptide of interest into the interstitial space of a tissue comprising the cell, whereby the naked polynucleotide is taken up into the interior of the cell and has a physiological effect. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* as well.

Compositions for use *in vitro* and *in vivo* comprising a "naked" polynucleotide are described in PCT application No. WO 90/11092 (Vical Inc.) and also in PCT application No. WO 95/11307 (Institut Pasteur, INSERM, Université d'Ottawa) as well as in the articles of Tacson et al. (1996) *Nature Medicine.* 2(8):888-892 and Huygen et al. (1996) *Nature Medicine.* 2(8):893-898.

In still another embodiment of the invention, the transfer of a naked polynucleotide of the invention, including a polynucleotide construct of the invention, into cells may be proceeded with a particle bombardment (biolistic), said particles being DNA-coated microprojectiles accelerated to a high

velocity allowing them to pierce cell membranes and enter cells without killing them, such as described by Klein et al. (1987) *Nature*. 327:70-73.

In a further embodiment, the polynucleotide of the invention may be entrapped in a liposome (Ghosh and Bacchawat, 1991, *Targeting of liposomes to hepatocytes*, IN : *Liver Diseases, Targeted diagnosis and therapy using specific receptors and ligands*. Wu et al. Eds., Marcel Dekeker, New York, pp. 87-104; Wong et al., 1980, Gene, 10 : 87-94; Nicolau C. et al. (1987), *Methods Enzymol.* 149:157-76). These liposomes may further be targeted to cells expressing LSR by incorporating leptin, triglycerides, Acrp30, or other known LSR ligands into the liposome membrane.

In a specific embodiment, the invention provides a composition for the *in vivo* production of a leptin polypeptide, or a zinc finger protein, described herein. It comprises a naked polynucleotide operatively coding for this polypeptide, in solution in a physiologically acceptable carrier, and suitable for introduction into a tissue to cause cells of the tissue to express the said polypeptide.

The amount of vector to be injected to the desired host organism varies according to the site of injection. As an indicative dose, it will be injected between 0,1 and 100 µg of the vector in an animal body, preferably a mammal body, for example a mouse body.

In another embodiment of the vector according to the invention, it may be introduced *in vitro* in a host cell, preferably in a host cell previously harvested from the animal to be treated and more preferably a somatic cell such as a muscle cell. In a subsequent step, the cell that has been transformed with the vector coding for the desired leptin polypeptide or the desired fragment thereof is reintroduced into the animal body in order to deliver the recombinant protein within the body either locally or systemically.

VI. Recombinant Cells of the Invention

The invention is in part based on the surprising and unexpected discovery that the different subunits of LSR interact to form at least two very different receptors : LSR-lep and LSR-tg. The LSR-lep receptor requires at least α'. In some embodiments a combination with β and/or α as well as α' is preferred. The LSR-tg receptor requires a combination of at least α and β. In some embodiments a combination with β and/or α as well as α' is preferred. Based on this novel and unexpected finding, it has become critical to engineer cells lacking endogenous LSR activity/expression (*e.g.* as a result of a classical knock-out, chimeraplasty, or zinc finger protein inhibition), and then to re-transfect the subunits of interest in various combinations and at various levels. This will allow not only the study of these receptors in isolation, but also the design of specific inhibitors for the different receptors, and the assessment of what genes may act to regulate or modulate the receptors, or to transmit the intracellular signals from or for each receptor. Although LSR-lep and LSR-tg have been identified, it is possible that other LSR receptors with other activities also exist and can be identified by these methods.

Recombinant cells have been designed that are useful in many situations, including : (1) the study of the role of the various LSR components in isolation and together with and without interference

from endogenous LSR, (2) as part of an assay system to discover modulators of the leptin/LSR interaction, for example, using known components of the LSR system (and in some cases no endogenous LSR components; see above), and (3) to produce various polypeptides of the invention (see above). To this end, in preferred embodiments, a recombinant cell is transiently, or preferably stably, transfected with one or more LSR subunits selected from the group consisting of α, α' and β. Preferably, the two or more subunits are expressed in pairwise ratios to each other of from 1:1 to 1:5. For example, if α and β are present in a cell, cells with ratios of 1 :1, 1 :2, 1 :3, 1 :4, 1 :5, 5 :1, 4 :1, 3 :1, 2 :1, as well as 2 :3, 3 :2, 3 :4, 4 :3, 3 :5, 5 :3, 4 :5, and 5 :4, etc. are preferred. Similar ratios are desired for cells containing α' and β. When all three subunits are present, cells with all possible combinations of ratios are preferred. These are most easily obtained by screening cells (wild-type, transfected, or knockout, for example) for their expression levels of the various subunits. Preferably, the one or more LSR components are α' and β, and preferably the recombinant cells are cultured PLC cells. However, the cells can be selected from any of the cells in the ATCC bank. The LSR polypeptides, the polynucleotides encoding LSR, and the vectors to transfer the polynucleotides encoding LSR between cells and tissues have been described previously (US National phase application No. 09/269,939, hereby incorporated herein by reference in its entirety including any figures, drawings or tables).

Another object of the invention consists of host cells that have been transformed or transfected with one of the polynucleotides described herein, and more precisely a polynucleotide comprising: a polynucleotide encoding a leptin polypeptide of the invention, or a polynucleotide encoding a zinc finger protein of the invention. These polynucleotides can be present in the same cell or in a different cell, and can be present in cells transiently or stably transfected with any combination of the components of LSR.

In another embodiment, the invention features cells that lack expression of at least one of the LSR subunits. These can be cells identified by screening processes, but they are preferably recombinant cells that have had the gene for LSR knocked-out by traditional techniques well known in the art; a cell in which a polynucleotide encoding a zinc finger protein of the invention has been transfected that either constitutively suppresses the expression of at least one subunit of LSR or whose suppression of LSR can be regulated by the Tet On/Off system, for example; or a cell in which the expression of at least one subunit of LSR has been inhibited as the result of the transfection of chimeric oligonucleotides of the invention.

The invention further features either transiently, or preferably stably, transfecting the LSR knockout cells (or zinc finger protein cells) in which expression of at least one, and in some cases all, of the endogenous LSR subunits has been inhibited (or eliminated), with at least one, preferably at least two, and alternatively three, of the LSR subunits and then selecting/screening for cells expressing the various ratios of subunits as described above. Preferably, β, α or α' alone are transfected, or alternatively α' and β, or α and β together are transfected.

The invention includes host cells that are transformed (prokaryotic cells) or that are transfected (eukaryotic cells) with a recombinant vector such as any one of those described in « Recombinant Vectors of the Invention ».

Generally, a recombinant host cell of the invention comprises at least one of the polynucleotides or the recombinant vectors of the invention which are described herein, but also includes those cells in which the gene for LSR has been knock-out by traditional recombinant techniques, zinc finger techniques, or using chimeraplast oligonucleotides.

Preferred host cells used as recipients for the recombinant vectors of the invention are the following :

a) Prokaryotic host cells : *Escherichia coli* strains (*i.e.* DH5-α strain), *Bacillus subtilis*, *Salmonella typhimurium*, and strains from species like *Pseudomonas*, *Streptomyces* and *Staphylococcus*, and

b) Eukaryotic host cells : HeLa cells (ATCC N°CCL2; N°CCL2.1; N°CCL2.2), Cv 1 cells (ATCC N°CCL70), COS cells (ATCC N°CRL1650; N°CRL1651), Sf-9 cells (ATCC N°CRL1711), C127 cells (ATCC N° CRL-1804), 3T3 (ATCC N° CRL-6361), CHO (ATCC N° CCL-61), human kidney 293 (ATCC N° 45504; N° CRL-1573), BHK (ECACC N° 84100501; N° 84111301), PLC cells, HepG2, Hepa 1-6, and Hep3B.

The constructs in the host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence.

Following transformation of a suitable host and growth of the host to an appropriate cell density, the selected promoter is induced by appropriate means, such as temperature shift or chemical induction, and cells are cultivated for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in the expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known by the skilled artisan.

Further, according to the invention, these recombinant cells can be created *in vitro* or *in vivo* in an animal, preferably a mammal, most preferably selected from the group consisting of mice, rats, dogs, pigs, sheep, cattle, and primates, not to include humans. Recombinant cells created *in vitro* can also be later surgically implanted in an animal, for example. Methods to create recombinant cells *in vivo* in animals are well-known in the art, and are specifically meant to include the techniques associated with chimeraplasty described herein and known in the art, whereby the chimeraplast oligonucleotides are provided to the cells in the animal by the use of liposomes, preferably liposomes that have targeting molecules for cells containing LSR such as LSR binding proteins or ligands, such as apm1, C1q, or leptin, for example, in the membrane layer.

VIII. Assays for Identifying Modulators of LSR Activity

The surprising and unexpected discovery that the different subunits of LSR interact to form at least two very different receptors (LSR-lep and LSR-tg) with different activities has resulted in the necessity of designing novel assays to identify inhibitors for the different LSR receptors. In particular, these assays will preferably utilize the recombinant cells of the invention, that are engineered to lack endogenous LSR activity/expression (*e.g.* as a result of a classical knock-out, chimeraplasty, or zinc finger protein inhibition). These cells are then re-transfected with the subunits of interest in various combinations and at various levels. Preferred combinations include those that give rise to the LSR-lep receptor that requires at least α', but may also include combination of α' and β, and the LSR-tg receptor that requires a combination of α and β. Other combinations (and the individual subunits) are also useful to look for other LSR receptor activities and as controls for the activity of compounds (or genes) selected in the other assays.

The invention features methods of screening for one or more compounds that modulate LSR activity in cells, that includes providing potential compounds to be tested to the cells, and where modulation of LSR activity indicates the one or more compounds. In some preferred embodiments, the potential compounds are compounds that have been molecularly designed based on the identified fragment of leptin that binds and activates LSR as described herein.

In a preferred embodiment, the invention features a method for selecting a compound useful for the treatment or prevention of an obesity-related disease or disorder, comprising:
contacting a recombinant cell that comprises a zinc finger protein of the invention, or a recombinant vector comprising any of the zinc finger proteins of the invention with a candidate compound; and
detecting a result selected from the group consisting of a modulation of an activity of the Lipolysis Stimulated Receptor and modulation of expression of the Lipolysis Stimulated Receptor; as a means for selecting said compound useful for the treatment or prevention of said obesity-related disease or disorder.

In preferred embodiments, said contacting is in the presence of a ligand of said Lipolysis Stimulated Receptor. Preferably, said ligand is selected from the group consisting of cytokine, lipoprotein, free fatty acid, adipoQ (Apm1 and Acrp30), and C1q, and more preferably said cytokine is leptin. Alternatively, said free fatty acid is oleate. In other preferred embodiments, said leptin is a leptin polypeptide fragment that modulates the activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of any one of the leptin polypeptide sequences set forth in Figure 13, wherein said at least 4 and not more than 50 contiguous amino acids comprise the leptin fragment central sequence. In other preferred embodiments, said leptin is a variant of a leptin polypeptide fragment that modulates the activity of LSR, consisting of a 22 contiguous amino acid sequence that is at least 75% identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13. Optionally, the leptin fragment is any leptin fragment of the invention described herein.

In other preferred embodiments of the invention, said activity is selected from the group consisting of binding of lipoproteins, uptake of lipoproteins, degradation of lipoproteins, binding of leptin, uptake of leptin, and degradation of leptin. Preferably, said modulation of LSR activity is an increase in said activity, and optionally a decrease in said activity. In other preferred embodiments, said expression is on the surface of said cell, and preferably said detecting comprises FACS, more preferably said detecting further comprises antibodies that bind specifically to said LSR, wherein said LSR comprises an amino acid sequence at least 75% homologous to at least one of the sequences selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19. In other preferred embodiments, said amino acid sequence is at least 80, 85, 90, 95, or 99 to 100% homologous to at least one of the sequences selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19. In other preferred embodiments, said antibodies bind specifically to a region of said LSR selected from the group consisting of an amino terminus, a carboxy terminus, a splice site, a cytokine binding site, a fatty acid binding site, a clathrin binding site, an apoprotein ligand binding site, a LI/LL motif, a RSRS motif, and a hydrophobic region. Preferably, said cell is selected from the group consisting of PLC, CHO-K1, Hep3B, and HepG2, although any cell expressing detectable levels of LSR can be used.

Antibodies to LSR and to the various regions of LSR have been extensively described previously in US National application 09/269,939, filed May 28, 1999 and its related PCT application, both are hereby incorporated herein by reference in their entirety including any figures, drawings or tables. In addition, specific antibodies to LSR are described in the Examples (1-8).

In preferred embodiments, said candidate compound is selected from the group consisting of peptides, peptide libraries, non-peptide libraries, peptoids, fatty acids, lipoproteins, medicaments, antibodies, and small molecules, and optionally can include leptin mimetics designed by methods of the invention. The compounds may be active *in vitro* or *in vivo*. The activity may be increased or decreased ; the compounds may be antagonists or agonists.

Preferably, said obesity-related diseases and disorders are selected from the group consisting of obesity, anorexia, cachexia, cardiac insufficiency, coronary insufficiency, stroke, hypertenison, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, and hyperuricemia. The compounds may also modulate body mass. Most preferably, the diseases include congenital generalized lipodystrophy.

In other highly preferred embodiments of the invention, the cells used in the above-described assays cells have been modified to express none, or a subset, of the LSR subunits. The recombinant cells containing zinc finger proteins of the invention are also transfected with at least one polynucleotide encoding a LSR polypeptide comprising a sequence at least 75% identical to an amino acid sequence selected from the group consisting of .SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ

ID NO:11, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19. Preferably the LSR subunit is stably transfected. Preferably the cell is selected from the group consisting of PLC, CHO-K1, Hep3B, Hepa 1-6, and HepG2. However, other cells available from the ATCC, for example, may also be used. In addition, cells with the endogenous LSR gene « knocked out » by methods well-known in the are are also expressly contemplated (as an option to the use of the zinc finger proteins of the invention, or to the use of the chimeraplasts of the invention.). Cells, preferably modified cells, are transfected with one or more LSR components that may include one, part, or all, of α', α, and β, most preferably α' and β. Recombinant cells useful for assays to identify modulators of the leptin-LSR interaction include those described in the « Recombinant Cells of the Invention ». In particular, cells expressing a range of ratios of the subunits are desired, including 1 :1, 1 :2, 1 :3, 1 :4, 1 :5, 5 :1, 4 :1, 3 :1, 2 :1, as well as 2 :3, 3 :2, 3 :4, 4 :3, 3 :5, 5 :3, 4 :5, and 5 :4, etc. for α' to β or α to β, or even α to α', for example. In addition, the various combinations where all three subunits are present in a cell are also envisioned to be useful in assays for modulators of LSR activity.

In highly preferred embodiments of the invention, cells with endogenous LSR activity knocked-out and transfected with the α' alone, or α' and β LSR subunits together are used to screen for modulators of the LSR-leptin interaction. In other preferred embodiments, the α and β LSR subunits are used to screen for modulators of triglyceride-rich lipoprotein binding, uptake, and degradation. Cells with all three LSR subunits are useful to screen for modulators of the effect of leptin binding uptake and degradation on triglyceride-rich lipoprotein binding, uptake and degradation. Similarly, these cells would be useful for screening molecules arising from the active leptin fragment molecular modeling described herien.

IX. Methods for Designing Leptin Polypeptide Fragment Mimetics

Following the discovery of the differential results of human and mouse leptin on human and rodent LSR, the region of amino acid sequence sharing the least homology between the two homologs was identified and was found to stimulate rodent and human LSR activity differentially (Examples 1-8). Identification of the differences between these two highly similar peptides allows the design of small molecule activators or inhibitors of LSR. Methods of determining the differences are well known in the art and include, but are not limited to techniques such as molecular dynamic assays, X-ray crystallography, and NMR. Previously, these kinds of techniques for creating inhibitors/activators of enzymes have been used successfully in the art. Potential small molecule activators/inhibitors designed or identified by these methods can be tested in the assays described herein. Those that function in these assays can then be tested for their effectiveness for treatment of obesity-related disorders and diseases, as described herein, for activity in modulating body mass, and for activity in treating congenital generalized lipodystrophy (Example 14).

The invention features a method of designing mimetics of a leptin fragment that modulates an activity of LSR, comprising: identifying critical interactions between one or more amino acids of said leptin fragment and LSR; designing potential mimetics to comprise said critical interactions; and testing said potential mimetics ability to modulate said activity as a means for designing said mimetics. By « designing mimetics » as used herein is meant comparing and combining known molecules to obtain a molecule that is able to mimic some or all of the activities modulated by leptin, or to preferentially increase or decrease some of the activities normally modulated by leptin. These activities include, but are not limited to those activities selected from the group consisting of leptin binding, leptin uptake, leptin degradation, triglyceride binding, triglyceride uptake, and triglyceride degradation. The methods of comparing and combining use molecular modeling, X-Ray crystallography and other techniques well-known in the art to identify the critical interactions. These critical interactions include, but are not limited to those selected from the group consisting of hydrogen bonding, covalent bonding, Van der Waals forces, steric hindrances, and hydrophobic interactions. These critical interactions are identified using assays that include, but are not limited to, those selected from the group consisting of NMR, X-ray crystallography, and computer modeling. Preferably the non-leptin compounds that are identified or designed by these means include, but are not limited to, small molecules (molecular weight <500, alternatively between 500 and 1000 MW, or >1,000 MW), peptides, peptide libraries, non-peptide molecules, non-peptide libraries and peptoids.

In preferred embodiments, the leptin fragment to be mimicked consists of the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13, preferably the human or mouse sequence, most preferably the human sequence. In other embodiments, the leptin fragment consists of the leptin fragment central sequence of any one of the leptin polypeptide sequences set forth in Figure 13, preferably the human or mouse sequence, most preferably the human sequence.

Methods of studying the structure of enzyme-substrate complexes are well known in the art. X-Ray crystallography allows the determination of the precise three-dimensional positions of most of the atoms in a protein molecule. To do this, a source of x-rays, a protein crystal, and a detector are needed. Obtaining the crystal is necessary because the techniques requires that all the molecules are precisely positioned. Methods to produce crystals are well-known in the art. X-rays going through the protein crystal are scattered by electrons, thus the amplitude of the wave scattered by an atom is proportional to its number of electrons. The scattered waves then recombine, either reinforcing one another on the film or cancelling each other out, depending on the atomic arrangement. From this information, the image is formed by applying a mathematical relation called a Fourier transform, and from here an electron-density map can be calculated, and then interpreted. The limiting resolution for a protein with a good crystal is typically 2 A.

Two methods important for enzyme-ligand interactions include (1) the difference Fourier method, and (2) production of stable complexes. In the Fourier method, the enzyme is crystallized (in

this case LSR) and then the X-ray diffraction of the crystalized protein in solvent is compared with the X-ray diffraction of the crystallized protein in the presence of ligand (in this case the 22 amino acid leptin peptide). Provided that there are no drastic changes in the structure or packing of the protein when it binds the ligand, the structure of the complex can be solved by comparing the diffrences between the diffraction patterns. This allows the electron density of the bound ligand and minor changes in the protein structure to be obtained without starting from scratch.

Alternatively, the X-ray diffraction pattern of a stably bound complex can be used to determine the protein-ligand interactions. Sometimes this is done using an inhibitor of the ligand, but can also be achieved under unreactive conditions such as : (1) weakly reactive conditions due to pH conditions, ionic state, or very low temperature, (2) using a chemically modified protein or ligand in which important residues are modified, or (3) under conditions in which the equilibrium conditions are shifted.

X-ray crystallography can be complemented by nuclear magnetic resonance (NMR) spectroscopy, which can reveal the structure of macromolecules in solution. Certain atomic nuclei such as hydrogen are intrinsically magnetic. The spinning of the positively charged proton, generates a magnetic moment. This moment can take either of two orientations when an external magnetic field is applied. The flow of electrons around a magnetic nucleus generates a small local magnetic field that opposes the external field. Under different environments the energy is absorbed at different resonance frequencies, an effect termed a chemical shift. Comparison of the shifts and spin-spin couplings, as well as the nuclear Overhauser effect (NOESY spectra) leads to the identification of pairs of protons that are less than 5A apart. Overlapping peaks in NOESY spectra can be further resolved by obtaining NMR spectra of proteins labelled with 15N and 13C (multidimensional NMR spectroscopy). Typically highly concentrated solutions of proteins are required (1 mM or 15 mg/ml for a 15 kd protein) and the size is generally limited to 30 kd.

Molecular modelling by computer is also used extensively to augment, supplement and integrate the information gained by X-Ray crystallography, NMR, EPR and other techniques. In particular, computer programs such as DOCK allow the prediction, identification, and three-D testing of inhibitors and activators of enzymes. This methodology has been used successfully previously to identify inhibitors. Basically, using the information gained from X-ray crystallography, NMR, and direct modelling, computer programs can now predict the residues that are important for the ligand-protein interactions and can predict structures that can perform the same interactions and test compounds proposed to be able to perform the same interactions. Through this interplay, molecules can be designed and identified to activate LSR in the manner of the leptin peptide, or to inhibit this interaction. The advantages to designing a molecule in this way include the ability to use compounds that the body cannot metabolize as rapidly as a peptide, that are less expensive to make, and that hopefully lack any unwanted leptin-associated side-effects.

X. Pharmaceutical Compositions of the Invention

The identified compounds can be administered to a mammal, including a human patient, alone or in pharmaceutical compositions where they are mixed with suitable carriers or excipient(s) at therapeutically effective doses to treat or ameliorate a variety of disorders associated with lipid metabolism. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms of obesity-related diseases or disorders as determined by the methods described herein. Thus, a therapeutically effective dosage of a leptin polypeptide fragment of the invention, or an antagonist or agonist of the leptin-LSR interaction, or a leptin fragment mimetic designed from molecular modeling studies, will be that dosage of the compound that is adequate to promote reduced or increased triglyceride-rich lipoprotein levels following a high-fat meal and that will promote weight loss or weight gain with continued periodic use or administration. Similarly, a therapeutically effective dosage of a chimeric oligonucleotide of the invention or a polynucleotide encoding a zinc finger protein of the invention will be that dosage of the compound that is adequate to increase or reduce triglyceride-rich lipoprotein levels following a high-fat meal and that will promote weight loss or weight gain with continued periodic use or administration. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

Additional aspects of the invention feature the use of the compounds, chimeric oligonucleotides and zinc fingers, described throughout the application as modulators of LSR activity in the making of medicaments for the treatment of diseases and disorders described in the following section as well as throughout the application. These diseases or disorders include, but are not limited to, anorexia, cachexia, AIDS-related weight loss, neoplasia-related weight loss, or obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes. Modulators of body mass are also expressly included, as are compounds (such as the leptin fragments of the invention) for treating congenital generalized lipodystrophy.

Routes of Administration.

Suitable routes of administration include oral, rectal, transmucosal, or intestinal administration, parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal or intraocular injections. A particularly useful method of administering compounds for promoting weight loss involves surgical implantation, for example into the abdominal cavity of the recipient, of a device for delivering the compound over an extended period of time. Sustained release formulations of the invented medicaments particularly are contemplated.

Composition/Formulation

Pharmaceutical compositions and medicaments for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries. Proper formulation is dependent upon the route of administration chosen.

Certain of the medicaments described herein will include a pharmaceutically acceptable carrier and at least one polypeptide that is a leptin polypeptide of the invention. In addition to medicaments that include leptin polypeptides of the invention, non-protein compounds designed based on molecular modeling of the active leptin polypeptide of the invention also will find utility as modulators of LSR activity, both *in vitro* and *in vivo*. Further, antagonists and agonists of the leptin-LSR interaction, including leptin and/or triglyceride-rich lipoprotein binding, uptake and degradation will also find utility in modulating LSR activity and/or stimulating a reduction of plasma lipoproteins and/or promoting weight loss.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer such as a phosphate or bicarbonate buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Pharmaceutical preparations that can be taken orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable gaseous propellant, *e.g.,* carbon dioxide. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.,* gelatin, for use in an inhaler or insufflator, may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.,* by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.,* in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms

as suspensions, solutions or emulsions in aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Aqueous suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder or lyophilized form for constitution with a suitable vehicle, such as sterile pyrogen-free water, before use.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Effective Dosage.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve their intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes or encompasses a concentration point or range shown

to effect enhanced or inhibited LSR activity in an *in vitro* system. Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50, (the dose lethal to 50% of the test population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD5O and ED5O. Compounds that exhibit high therapeutic indices are preferred.

The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50, with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. *(See, e.g.,* Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound which are sufficient to maintain the LSR modulating effects. Dosages necessary to achieve the LSR modulating effect will depend on individual characteristics and route of administration.

Dosage intervals can also be determined using the value for the minimum effective concentration. Compounds should be administered using a regimen that maintains plasma levels above the minimum effective concentration for 10-90% of the time, preferably between 30-90%; and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

A preferred dosage range for the amount of a leptin polypeptide of the invention, or compound designed based on its molecular modeling, or an antagonist or agonist of its activity with LSR, that can be administered on a daily or regular basis to achieve desired results, including a reduction in levels of circulating plasma triglyceride-rich lipoproteins, range from 0.1 - 50 mg/kg body mass. A more preferred dosage range is from 0.2 - 25 mg/kg. A still more preferred dosage range is from 1.0 - 20 mg/kg, while the most preferred range is from 2.0 - 10 mg/kg. Of course, these daily dosages can be delivered or administered in small amounts periodically during the course of a day.

XI. Methods of Preventing or Treating Obesity-related Diseases and Disorders

A method of preventing or treating obesity-related diseases and disorders comprising providing a patient in need of such treatment with a leptin polypeptide fragment or a leptin mimetic of the invention. Preferably, the leptin polypeptide fragment or mimetic modulates the activity of LSR, more preferably increases the activity of LSR, and optionally decreases the activity of LSR either *in vitro* or *in vivo*. Preferably the leptin polypeptide fragment or mimetic is provided to the patient in a pharmaceutical composition that is preferably taken orally. Preferably the patient is a mammal, and most preferably a human. In preferred embodiments, the obesity-related disease or disorder is selected from the group consisting of anorexia, cachexia, AIDS-related weight loss, neoplasia-related weight loss, or obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes. Modulators of body mass (weight gain or loss) are also expressly included, as are compounds (such as the leptin fragments of the invention) for treating congenital generalized lipodystrophy.

Alternatively, the invention features a method of preventing or treating obesity-related diseases and disorders comprising providing a patient in need of such treatment with a compound identified by assays of the invention. Preferably these compounds antagonize or agonize the interaction of leptin and LSR. In other embodiments, the compounds are those created as a result of the molecular modeling of the active leptin polypeptide and are non-peptide mimetics that function in the same manner as the active leptin polypeptide of the invention. Preferably, the compound is provided to the patient in a pharmaceutical composition that is preferably taken orally. Preferably the patient is a mammal, and most preferably a human. In preferred embodiments, the obesity-related disease or disorder is selected from the group consisting of anorexia, cachexia, AIDS-related weight loss, neoplasia-related weight loss, or obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes. Modulators of body mass are also expressly included, as are compounds (such as the leptin fragments of the invention) for treating congenital generalized lipodystrophy.

The invention also features a method for treating or preventing obesity-related diseases or disorders involving gene surgery. To this end, it is advantageous in some conditions to either express more or less LSR, or alternatively to express more or less of one or more LSR subunits. Using the methods described herein, it is possible to modulate the levels of expression of LSR, or of some LSR subunits using zinc finger polypeptides of the invention or chimeric oligonucleotides of the invention. Preferably, the zinc finger polypeptides are provided to an individual in need of such treatment by polynucleotides encoding the zinc finger polypeptides of the invention. Preferably the zinc finger

polynucleotides of the invention are present in a recombinant vector, preferably a retroviral vector, more preferably AAV. Preferably the chimeric oligonucleotides are provided to a patient in need of such treatment using liposomes. Preferably the liposomes are constructed such that molecules targeting the liposomes to cells containing LSR are present in the membrane. Preferably the molecules include leptin, apm1, and C1q, for example. Alternatively they may have compounds that target them to the liver, such as glucose, for example, or alternatively to adipose tissue. Preferably the patient is a mammal and the obesity-related disease or disorder is selected from the group consisting of anorexia, cachexia, AIDS-related weight loss, neoplasia-related weight loss, or obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes. Modulators of body mass are also expressly included, as are compounds (such as the leptin fragments of the invention) for treating congenital generalized lipodystrophy.

Still another aspect of the invention relates to the use of chimeric oligonucleotides to specifically alter single nucleotide polymorphisms in a patient in need of such treatment. Single polymorphisms associated with the LSR gene and with obesity have been described in U.S. provisional application No. 60/119,592, entitled "Polymorphic Markers of the LSR gene" by Blumenfeld et al, filed February 10, 1999, which is hereby incorporated by reference herein in its entirety including any drawings, figures, or tables , and shown in Table A . In one embodiment, this medicament can be used for reducing food intake in obese individuals, reducing the levels of free fatty acids in obese individuals, decreasing the body weight of obese individuals, or treating an obesity related condition selected from the group consisting of obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes. Modulators of body mass are also expressly included, as are compounds (such as the leptin fragments of the invention) for treating congenital generalized lipodystrophy.

Table A

Biallelic Marker	**Marker Name**	**Localization In LSR Gene**	**Polymorphism**	**Frequency Of Allele 2**	**AA Change**	**Marker Position**
99-14410/373	A1	*5'regulatory region*	Allele 1: C Allele 2: T			373 of SEQ ID No 2
99-14424/353	A2	*5'regulatory region*	Allele 1: A Allele 2: G			353 of SEQ ID No 3
99-14418/322	A3	*5'regulatory*	Allele 1: A			322 of

		region	Allele 2: G			SEQ ID No 4
99-14417/126	A4	*5'regulatory region*	Allele 1: C Allele 2: T			126 of SEQ ID No 5
99-14417/334	A5	*5'regulatory region*	Allele 1: C Allele 2: T			334 of SEQ ID No 5
99-14415/106	A6	*5'regulatory region*	Allele 1: C Allele 2: T			106 of SEQ ID No 6
99-14413/250	A7	*5'regulatory region*	Allele 1: A Allele 2: C			250 of SEQ ID No 7
99-14413/383	A8	*5'regulatory region*	Allele 1: G Allele 2: T			383 of SEQ ID No 7
99-4575/226	A9	*5'regulatory region*	Allele 1: T Allele 2: C	25%		226 of SEQ ID No 8
9-19/148	A10	*5'regulatory region*	Allele 1: C Allele 2: T	15%		1243 of SEQ ID No 1
9-19/307	A11	*5'regulatory region*	Allele 1: A Allele 2: T	12%		1401 of SEQ ID No 1
9-19/442	A12	*5'regulatory region*	Allele 1: C Allele 2: Del C			1535 of SEQ ID No 1
9-20/187	A13	*5'regulatory region*	Allele 1: A Allele 2: C			1788 of SEQ ID No 1
9-1/308	A14	*Intron 1*	Allele 1: C Allele 2: G	24%		2391 of SEQ ID No 1
9-3/324	A15	*Exon2*	Allele 1: C Allele 2: T	29%		3778 of SEQ ID No 1; 595 of SEQ ID Nos 13, 15, and 17
99-14419/424	A16	*Intron 2*	Allele 1: C Allele 2: A	22%		4498 of SEQ ID No 1
9-24/260	A17	*Intron 3*	Allele 1: A Allele 2: G	35%		15007 of SEQ ID No 1
9-24/486	A18	*Intron 4*	Allele 1: G Allele 2: A	15%		15233 of SEQ ID No 1
9-6/187	A19	*Exon 5*	Allele 1: C	1%		15826 of

			Allele 2: T			SEQ ID No 1; 940 of SEQ ID No 13; 883 of SEQ ID No 15
9-7/148	A20	*Intron 5*	Allele 1: G Allele 2: A	35%		19567 of SEQ ID No 1
9-7/325	A21	*Exon 6*	Allele 1: G Allele 2: A	14%	S→N	19744 of SEQ ID No 1; 1191 of SEQ ID No 13; 1134 of SEQ ID No 15; 987 of SEQ ID No 17
9-7/367	A22	*Intron 6*	Allele 1: A Allele 2: C			19786 of SEQ ID No 1
9-9/246	A23	*Exon 8*	Allele 1: C Allele 2: G	0.5%	P→R	20158 of SEQ ID No 1; 1362 of SEQ ID No 13; 1305 of SEQ ID No 15; 1158 of SEQ ID No 17
LSRX9-BM (17-1/240)	A24	*Exon 9*	Allele 1: AGG Allele 2: Del AGG	Del 26%	Del R	20595 of SEQ ID No 1; 1658 of SEQ ID No 13; 1601 of SEQ ID No 15; 1454 of SEQ ID No 17
LSRX10-BM	A25	*Exon 10*	Allele 1 : T Allele 2 : G			21108 of SEQ ID No 1; 2079 of

						SEQ ID No 13; 2022 of SEQ ID No 15; 1875 of SEQ ID No 17
99-4580/296	A26	*3'regulatory region*	Allele 1 : A Allele 2 : G	24%		296 of SEQ ID No 9
99-4567/424	A27	*3'regulatory region*	Allele 1 : C Allele 2 : T			424 of SEQ ID No 10
99-14420/477	A28	*3'regulatory region*	Allele 1 : G Allele 2 : T			477 of SEQ ID No 11
99-4582/62	A29	*3'regulatory region*	Allele 1 : A Allele 2 : G			62 of SEQ ID No 12
99-4582/359	A30	*3'regulatory region*	Allele 1 : G Allele 2 : T	24%		359 of SEQ ID No 12
17-2/297	A31	*5' regulatory region*	Allele 1 : C Allele 2 : G	48%		818 of SEQ ID No 1
9-19/256	A32	*5' regulatory regio*	Allele 1 : A Allele 2 : G			1374 of SEQ ID No 1

XII: Methods for Selecting Genes that Modulate LSR Expression

Another aspect of the invention features a method for selecting for genes that modulate the expression of LSR. This method relies on the use of a retroviral vector to provide cells of choice (those that express LSR naturally or recombinantly, and in any combination of subunits and subunit levels) with genes of interest at a moderate level. By « a moderate level » is meant a level that is intermediary between high and low, as based on the level of expression of GFP. Neither high nor low expression is desired since low levels might result in undetectable effects on LSR activity and high levels might co-opt the use of the cell machinery such that LSR isn't made simply for this reason. These moderate levels are easily detected and selected for by FACS analysis as described in the Examples. This method also relies on the use of FACS to detect changes in the activity of LSR as judged by detecting the expression of LSR, or LSR subunits on the surface of the cells, or alternatively intracellularly as well. This can be done by using two antibodies that bind specifically to different regions of LSR, for example the 81B and 93A antibodies.

Thus, in a preferred embodiment, the invention features a method of selecting for genes that modulate an activity of the Lipolysis Stimulated Receptor, comprising : providing a retroviral gene

library to cells that express said Lipolysis Stimulated Receptor; contacting said cells with a ligand of said Lipolysis Stimulated Receptor ; and detecting a change in said activity of the Lipolysis Stimulated Receptor as a means for selecting for said genes. Preferably, said retroviral gene library comprises a cDNA library from tissues selected from the group consisting of liver, brain, muscle, and adipose, and preferably further comprises a detectable marker protein selected from the group consisting of GFP, truncated CD2, and truncated CD4. In preferred embodiments, the method further comprises selecting said cells transfected with the retroviral vector for moderate expression of GFP. Preferably, said selecting of cells is by FACS.

In other preferred embodiments, said ligand is selected from the group consisting of cytokine, free fatty acid, lipoprotein, adipoQ (Acrp30, Apm1), and C1q, and preferably said cytokine is leptin. Preferably said free fatty acid is oleate. More preferably, said leptin is a leptin polypeptide fragment that modulates the activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of any one of the leptin polypeptide sequences set forth in Figure 13, wherein said at least 4 and not more than 50 contiguous amino acids comprise the leptin fragment central sequence. Optionally, said leptin is a variant of a leptin polypeptide fragment that modulates the activity of LSR, consisting of a 22 contiguous amino acid sequence that is at least 75% identical to the leptin fragment variable region of any one of the leptin polypeptide sequences set forth in Figure 13.

In other preferred embodiments, said detecting a change in said activity is by FACS, preferably said detecting further comprises fluorescent antibodies that bind specifically to said LSR, wherein said LSR comprises an amino acid sequence at least 75% homologous to at least one of the sequences selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16. More preferably, said antibodies bind specifically to a region of said LSR selected from the group consisting of an amino terminus, a carboxy terminus, a splice site, a cytokine binding site, a fatty acid binding site, a clathrin binding site, an apoprotein ligand binding site, a LI/LL motif, a RSRS motif, and a hydrophobic region.

Antibodies to LSR and to the various regions of LSR have been extensively described previously in US National application 09/269,939, filed May 28, 1999 and its related PCT application, both are hereby incorporated herein by reference in their entirety including any figures, drawings or tables. In addition, specific antibodies to LSR are described in the Examples (1-8).

In other preferred embodiments said cell is selected from the group consisting of PLC, CHO-K1, Hep3B, and HepG2. In some of these embodiments, said cell has had the endogenous LSR activity inhibited by either a traditional « knockout » of the gene encoding LSR, alternatively said cell has had the expression of endogenous LSR inhibited by transfection of a polynucleotide encoding a zinc finger protein of the invention, or by providing a chimeric oligonucleotide of the invention to the cell.

Other characteristics and advantages of the invention are described in the Brief Description of the Figures and the Examples. These are meant to be exemplary only, and not to limit the invention in any way.

Throughout this application, various publications, patents and published patent applications are cited. The disclosures of these publications, patents and published patent specifications referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show the effect of leptin on postprandial plasma TG response in db/db and db^{Pas}/db^{Pas} mice. Overnight-fasted *db/db* (A), db^{Pas}/db^{Pas} (B) mice were gavage-fed a high-fat test meal and immediately injected intravenously (*db/db*) or intraperitoneally (db^{Pas}/db^{Pas}) with saline (open symbols) or 50 μg mouse recombinant leptin (closed symbols). At the indicated times, blood was collected from the tail (A) or orbital (B) vein, plasma was separated by centrifugation, and plasma TG concentrations were determined using an enzymatic kit. Each point represents the mean ± SEM (*db/db*: saline, n = 4, leptin, n = 3; db^{Pas}/db^{Pas}: saline, n = 6, leptin, n = 7). The average plasma lipid response in 10 control C57BL6 mice is shown as a dotted line in both A and B. In a separate experiment, shown as an inset for each strain of mice, overnight fasted *db/db* (●) or db^{Pas}/db^{Pas} (■) mice were gavage-fed the test meal and immediately injected intravenously with increasing concentrations of leptin. The plasma lipid response was then measured as in A and B. The area under the response curve (AUC) was then calculated using a triangulation method on Microsoft Excel between 0 and 4 hr (mg TG · h / mL). Values are presented as % of control value (test meal alone obtained in A or B). Each point represents the mean of at least 3 mice.

Figure 2 shows ^{125}I-Leptin binding to partially purified rat LSR. Aliquots (72 μg) of partially purified rat liver LSR were separated on a 4%-12% SDS-gradient polyacrylamide gel, and transferred to nitrocellulose as described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398) a gel strip stained with Coomassie blue is shown in lane 1. The nitrocellulose strips were washed, blocked, and incubated with anti-rat LSR protein antiserum (1:1000 dilution)(lane 2), or with 200 ng/mL ^{125}I-leptin (lane 3). The strips were washed and bands were detected as described herein. Image analysis of lane 3 was performed on a Phosphorimager (Molecular Dynamics).

Figures 3A, 3B, 3C, and 3D show the effect of LSR subunit transfection on leptin binding and degradation in CHO-K1 cells. For Fig. 3A, CHO-K1 cells were transfected with increasing concentrations of the α, (□), α'(■) or β (○) LSR plasmid, or vector alone (●) using Fugene transfection reagent. After 48 h, the cells were washed once in PBS and incubated at 37 °C for 2 h with 10 ng/mL of ^{125}I-leptin in DMEM containing 0.2% (w/v) BSA, 2 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 (Buffer A). The monolayers were washed and lysed with 0.1 N NaOH containing 0.24 mM EDTA, and the lysates

were counted. The results are shown as the amount of cell-associated ^{125}I-leptin. For Fig. 3B, lysates were prepared from CHO-K1 wild type, stable transfectants of vector or LSR α' subunit, and PLC, and separated on a 10% SDS-polyacrylamide gel under denaturing and reduced conditions. After transfer to nitrocellulose, Western blots were performed using anti-LSR 170 antibody (can also be done with the human equivalent, 93A). Northern blots were done to detect LSR mRNA in CHO-K1 wild-type versus PLC. RT-PCR analysis was also done in CHO-K1 as compared to PLC. For Fig. 3C and 3D, confluent monolayers of stable-transfected cell lines expressing LSR α' subunit (■) or vector alone (●) were washed once in PBS and incubated at 37 °C for 2 h with increasing concentrations of ^{125}I-leptin in Buffer A. The amount of cell-associated (Fig. 3C) and degraded (Fig. 3D) ^{125}I-leptin was then measured as described herein. Results are shown as the mean of triplicate determinations.

Figures 4A, 4B, 4C, and 4D show LSR binding and degradation of ^{125}I-leptin in human hepatocytes, and the effect of 81B anti-LSR antibody. For Fig.4A, PLC cells were lysed (3-T175 cm^2 flasks per condition) and immunoprecipitated with irrelevant or 81B anti-serum. The immunoprecipitates were washed, were separated on 10% SDS-polyacrylamide gels under nondenaturing conditions, and were transferred to nitrocellulose. Ligand blots using ^{125}I-leptin were then performed as described in Figure 2. For Fig. 4B, confluent monolayers of PLC cells were incubated at 37 °C for 30 min with 100 nM insulin, were washed, and then were incubated for 30 min at room temperature in the presence of anti-LSR peptide 81B antibody (■), or irrelevant (□) IgG. After this, the cells were incubated at 37 °C for 2 h with increasing concentrations of ^{125}I-leptin in Buffer A. The monolayers were washed, and the amount of ^{125}I-leptin degraded was determined as described herein. Results are shown as the mean of duplicate (irrelevant IgG) or triplicate (anti-LSR peptide IgG) determinations. Fig. 4C is a schematic diagram of the motifs found from the predicted protein sequence of LSR α cDNA. A corresponding Kyte-Doolittle hydrophilicity plot (Lasergene, DNAstar, Madison, WI) is shown underneath. For Fig. 4D, PLC cell aliquots were prepared and incubated with irrelevant, 93A or 81B antibodies. After washing and incubation with goat-anti-rabbit FITC-conjugated antibody, the cells were fixed and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Figures 5A, 5B, 5C, and 5D show the stimulatory effect of leptin on LSR activity in PLC and suppression of this effect by 81B antibody. For Fig. 5A, 5B, and 5C, cultured PLC cells were incubated at 37 °C for 30 min with increasing concentrations of human recombinant leptin in Buffer A. After this, 0.5 mM oleate and 20 μg/mL ^{125}I-LDL were added, and cells were further incubated at 37 °C for 2 h. Cells were washed, and the amount of oleate-induced ^{125}I-LDL bound (5A), internalized (5B) and degraded (5C) were measured. For Fig. 5D, PLC cells were incubated at room temperature for 30 min with 200 μg/mL anti-LSR peptide 81B or 170 antibody, followed by incubation at 37 °C for 30 min without (open bar) or with (hatched bar) 10 ng/mL human leptin. Oleate (0.5 mM) and ^{125}I-LDL (20 μg/mL) were added, and the monolayers were left at 37 °C for 3 h. After washing, the amount of ^{125}I-LDL binding was determined, and is shown here as the mean ± SD of triplicate determinations.

Figures 6A, 6B, and 6C show the effect of leptin on ^{125}I-LDL and ^{125}I-chylomicron binding to LSR in primary cultures of rat hepatocytes. For Fig. 6A, primary cultures of rat hepatocytes (48 hours after plating) were incubated at 37 °C for 30 min in the absence (□) or presence (■) of 50 ng/mL leptin in Buffer A, followed by a 20 min incubation at 37 °C with 0.5 mM oleate. The cells were then washed with ice-cold PBS, precooled for 10 min, and then incubated for 1 h at 4 °C with increasing concentrations of ^{125}I-LDL in Buffer A. Cells were washed, were lysed in 0.1 N NaOH and were counted for radioactivity. Results are shown as the mean of duplicate determinations. For Fig. 6B, primary cultures of rat hepatocytes were incubated at 37 °C for 30 min with or without 20 ng/mL leptin followed by incubation at 37 °C for 4 h with 6 μg protein/mL ^{125}I-chylomicrons in the absence or presence of 0.5 mM oleate in Buffer A. The cells were then washed and the ^{125}I-chylomicrons bound to the cell surface were released into the media by incubation with 10 mM suramin. The media was recovered and the radioactivity was measured. Results are shown as the mean ± SD of six determinations. Fir Figure 6C, after incubation at 37 °C for 30 min with 50 ng/mL leptin, the cells were incubated at room temperature for 30 min with 200 μg IgG/mL antibodies directed against rat LSR protein or irrelevant IgG. The amount of ^{125}I-chylomicrons bound was determined, and results are shown as means ± SD of triplicate (irrelevant) or quadruplicate (anti-LSR) determinations.

Figures 7A and 7B show a comparison of the effect of human and mouse leptin on LSR activity in rat hepatocytes and on postprandial increase in plasma TG in db^{Pas} /db^{Pas} mice. For Fig. 7A, primary cultures of rat hepatocytes were incubated 30 min at 37 °C without (open bar) or with 10 ng/mL recombinant human (solid bar) or mouse (hatched bar) leptin in Buffer A. Oleate (0.5 mM) and ^{125}I-LDL (20 μg/mL) were added and the cells were incubated 2 h at 37 °C. The media were removed and were analyzed for TCA-soluble degradation products. The mean of duplicate determinations is shown. For Fig. 7B, *db^{Pas} /db^{Pas}* mice were given a test meal as previously described, followed immediately by injection *i.p.* of saline (open bar, n = 4), human leptin (1 μg/animal; solid bar, n = 3) or mouse leptin (0.25 μg/animal; hatched bar; n = 3). The data represent the difference in TG concentrations measured at t=0 and the average of the concentrations at 3 and 4 hours. Results are shown as mean ± SEM.
Figures 8A and 8B show the effect of mouse or human leptin on LSR activity in primary cultures of rat hepatocytes or a human liver cell line (PLC). Primary cultured rat hepatocytes were obtained commercially (In Vitro Tech). The PLC cell line was obtained from ATCC repository and maintained in culture. Rat hepatocytes 72 h after plating (8A) or confluent monolayers of PLC (8B) were incubated 30 min at 37 °C with 0 (closed bar) or 10 ng/mL of human (open bar) or mouse (hatched bar) recombinant leptin. Following this, 0.5 mM oleate and 20 μg/mL ^{125}I-LDL were added and the cells were further incubated for 2 h at 37 °C. The cells were washed, and the amount of oleate-induced ^{125}I-LDL bound, internalized and degraded was measured as described previously (Bihain, B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low-density lipoproteins in fibroblasts from a subject

homozygous for familial hypercholesterolemia. Biochemistry *31*, 4628-4636.. Results here are shown as % of control values obtained in the absence of leptin. Figure 9 shows the effect of mouse or human leptin peptide on LSR activity in PLC. Confluent PLC monolayers were incubated 30 min at 37 °C with increasing concentrations of mouse (■) or human (●) leptin peptide. Following this, 0.5 mM oleate and 20 μg/mL ^{125}I-LDL were added and the cells were further incubated for 2 h at 37 °C. The cells were washed, and the amount of oleate-induced ^{125}I-LDL bound and degraded was measured as described previously (Bihain, B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low density lipoproteins infibroblasts from a subject homozygous for familial hypercholesterolemia. *Biochemistry 31*, 4628-4636.

Figure 10 shows the effect of mouse or human leptin peptide on LSR activity in primary cultured rat hepatocytes. Cells were incubated 30 min at 37 °C with increasing concentrations of mouse (■) or human (●) leptin peptide. Following this, 0.5 mM oleate and 20 μg/mL ^{125}I-LDL were added and the cells were further incubated for 2 h at 37 °C. The cells were washed, and the amount of oleate-induced ^{125}I-LDL bound and degraded was measured as described previously (Bihain, B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low-density lipoproteins in fibroblasts from a subject homozygous for familial hypercholesterolemia. *Biochemistry 31*, 4628-4636).

Figures 11A and 11B show the effect of mouse leptin (Fig. 11A) or leptin peptide (Fig. 11B) on postprandial plasma TG response in ob/ob mice. A single dose of 50 ng mouse leptin (A, ■), leptin peptide (B, ■), or a comparable volume of saline (□) was injected subcutaneously at t = 0 h (8:30 AM) directly following gavage of a high fat meal (0.5 mL). Postprandial triglyceridemia was measured as described previously. Each point represents the mean ± SEM (A: saline, n = 8, leptin, n = 7; leptin peptide: saline, n = 8, leptin, n = 8). The insert to figure 11B shows the effect of mouse leptin on LSR activity in primary cultures of rat hepatocytes. Primary cultures of rat hepatocytes were incubated 30 min at 37 °C with increasing concentrations of mouse leptin peptide. Oleate (0.5 mM) and ^{125}I-LDL (20 μg/mL) were added and the cells were incubated 2 h at 37 °C. After washing, the amount of 125I-LDL bound to the cell surface was measured as described in the Examples section herein.

Figure 12 contains a Table that presents results showing the effect of test meal with and without leptin injection on postheparin lipolytic activity in db^{Pas}/db^{Pas} mice. Animals were gavaged with the test-meal and injected at the same time with 50 μg leptin or physiological saline as described previously (t = 0 h). After 1 h, the mice were injected with heparin and blood samples were taken at the peak of postprandial triglyceridemia (t = 2 h). Lipase activity was measured in the postheparin plasma as described in the Examples section herein,, and is reported here as the mean ± SEM (n = 3 animals for each condition; ns = not significant).

Figure 13 shows a multiple sequence alignment of leptin polypeptides from various species including: *Homo sapiens* (SEQ ID NO:32), *Mus musculus* (SEQ ID NO:34), *Rattus norvegicus* (SEQ ID NO:38), *Sus scrofa* (SEQ ID NO:39), *Bos Taurus* (SEQ ID NO:28), *Gallus gallus* (SEQ ID NO:30), *Ovus aries* (SEQ ID NO:35), *Canis familiaris* (SEQ ID NO:29), *Gorilla gorilla gorilla* (SEQ ID NO:31), *Macaca mulatta* (SEQ ID NO:33), *Pan troglodytes* (SEQ ID NO:36), and *Pongo pygmaeus* (SEQ ID NO:37). Divergent residues (from the consensus sequence) are boxed. The 22 amino acid region of the exemplary active leptin peptide is shaded for all species in the alignment. Residues 10-13 of the shaded region make up the "leptin fragment central sequence".

Figure 14 shows a schematic diagram of an exemplary retroviral vector. The vector pMX-IRES-GFP contains the murine Moloney virus LTR and a packaging signal (Onishi *et al.* Exp. Hematol. 24: 324-329 (1996)); the EMCV IRES is placed between the polylinker/stuffer and a cDNA encoding a selectable marker protein. Three exemplary marker proteins are GFP, murine CD2 and human CD4. The IRES sequence is indicated as a shaded box with an arrow indicating the direction of translation. The segment containing the bacterial origin of replication and ampicillin resistant gene is indicated by a black box. The stippled box represents sequence encoding the green fluorescent protein; alternatively it can contain the truncated CD2 or CD4 sequences. Open boxes with arrows indicate the viral LTR sequences. The open box indicates a stuffer fragment containing multiple cloning sites.

Figure 15 shows a schematic of a plan to create truncated LSR constructs.

Figures 16A, 16B, and 16C show that the transfection of a truncated form of LSR (DN5 + 6) increases ^{125}I-LDL binding (A), uptake (B) and degradation (C) in PLC cells in reference to protein concentration. All points are done in triplicate.

Figures 17A, 17B, and 17C show that the transfection of a truncated form of LSR (DN5+6) increases ^{125}I-LDL binding (A), uptake (B) and degradation (C) in PLC cells correcting for transfection efficiency using β-gal as a reference. All points are done in triplicate.

Figures 18A and 18B show graphs of the total LSR expression in mouse liver determined by Quantitative PCR.

Figures 19A and 19B show graphs of the expression of LSR isotypes in mouse liver.

Figures 20A and 20 B show graphs of the relative levels of LSR isotype expression in mouse liver.

Figure 21 shows a graph of total LSR expression in mouse brain determined by Quantitative PCR.

Figure 22 shows a graph of the expression of LSR isotypes in mouse brain.

Figure 23 shows a graph of the relative levels of LSR isotype expression in mouse brain.

Figures 24A, 24B, 24C, 24D, and 24E show the difference in LSR expression and activity in 2 cultured human hepatocyte cell lines. Figures 24A and 24B show graphs of LSR mRNA levels (24A) and cell surface expression (24B) in PLC (GG) and HepG2 (AG) cells by quantitative PCR and FACS,

respectively. Figures 24C, 24D, and 24E show graphs of the oleate-induced ^{125}I-LDL bound (A), internalized (B), and degraded (C) in confluent monolayers of PLC (■) and HepG2 (▲) that were incubated 3 h at 37 °C with the indicated concentrations of oleate and 20 μg/mL ^{125}I-LDL. The cells were then washed and the amounts of ^{125}I-LDL bound, internalized and degraded were measured as described previously.

Figure 25 shows a table of the characteristics of recombinant ZFPs directed toward LSR sequences. The first column is the identification number of the plasmid expressing a specifically engineered ZFP. The ZFP column represents different zinc finger "cassettes" designed to recognize the 9 bp regions of the target sequence. These "cassettes" have then been linked together (see WO 98/54311) to create the ZFP for the final 18 bp target sequence listed in the final column. Sangamo determined the data on the fold activation and binding constant. The target sequences are located 5' to the translation start site in the mouse LSR gene sequence.

Figures 26A, 26B, 26C, 26D, 26E, 26F, 26G, 26H, 26I, 26J, 26K, 26L, 26M, 26N, 26O, 26P, 26Q, 26R, 26S, and 26T show schematics and nucleotide sequence of the LSR zinc finger plasmids pSBS5182-NVF (26A), pSBS5183-NVF (26B), pSBS5185-NVF (26C), pSBS5186-NVF (26D), and pSBS5205-NVF (26E). The locations of the ampicillin gene (Amp), neomycin gene (Neo) CMV promoter NLS, ZFP, VP16, FLAG, bGHpA as well as various restriction sites are shown in the schematics.

Figure 27 shows a Northern Analysis of LSR zinc finger mRNA expression. Numbers are shown as percent of control plasmid. Only the results from 48 hrs are shown.

Figure 28 shows a more detailed Northern analysis of LSR zinc finger mRNA expression. Numbers are shown as percent of control plasmid. Only the results from 48hrs are shown.

Figure 29 shows a quantitative PCR Analysis of Hepa1-6 cells transfected with ZFP-NVF constructs.

Figures 30A, 30B, 30C, 30D, 30E, and 30F show binding, uptake and degradation (BUD) data from ZFPs. The following ZFP's were examined: 5185-NVF, 5186-NVF, and control plasmid VegF-NVF (a non related ZFP). Results are corrected for total protein in A-C and for β-gal in D-F.

Figure 31 shows a diagram of the coculture system. Endothelial cells are plated in the upper compartment on the filter and astrocytes in the lower compartment on the plastic of the Petri dish.

Figure 32 shows a diagram of transcytosis and permeability studies.

Figure 33 shows a graph of leptin transcytosis in BBB *in vitro* model. Cells were incubated with ^{125}I-leptin alone (10,000 dpm/ng)(closed squares), with 1 μg/mL unlabelled leptin (triangles), 50 μg/mL MP (circles), 50 μg/mL HP (open squares), or 2 mg/mL lactoferrin (asterisks).

Figure 34A and 34B show graphs of the effect of leptin, MP, HP, and lactoferrin on the permeability of the EC monolayer. Sucrose (34A) and inulin (34B) permeability studies were performed in the absence (diamonds) or presence of 10 ng/mL leptin (squares), 5 μg/mL leptin (triangles), 10

μg/mL leptin (crosses). The effect of peptides were also tested by the addition of 10 ng/mL leptin + 50 μg/mL mouse peptide (MP, circles) or 10 ng/mL leptin+50 μg/mL human peptide (HP, open squares) or 10 ng/mL leptin+2 mg/mL lactoferrin (lacto, asterisks).

Figures 35A and 35B show graphs of LSR activity and mRNA expression measured in PLC cells preincubated 24 h with leptin. In Figure 35A, PLC monolayers were incubated 24 h at 37 °C with (o) or without (n) 200 ng/mL human recombinant leptin. After washing with PBS, cells were incubated 30 min at 37 °C with increasing concentrations of human leptin, followed by a 2 h incubation at 37 °C with 0.8 mM oleate and 20 μg/mL ^{125}I-LDL. Cells were washed, and the amount of oleate-induced ^{125}I-LDL binding was measured as described previously. Results are shown as the mean of triplicate determinations. In Figure 35B, PLC monolayers were incubated 24 h at 37 °C with 0, 200, or 400 ng/mL human recombinant leptin. After washing with PBS, the cells were harvested. Total RNA was prepared from the cell pellets, and Northerns were performed to detect LSR mRNA, using GAPDH probe as loading control as described previously. Northern blots were scanned on the Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis of the images was performed using the software ImageQuant. Results are shown as the amount of LSR signal relative to that of GAPDH (mean ± SD, n = 3/condition).

EXAMPLES

The following Examples are provided for illustrative purposes and not as a means of limitation. One of ordinary skill in the art would be able to design equivalent assays and methods based on the disclosure herein all of which form part of the instant invention.

GENERAL MATERIALS and METHODS

Materials

Na^{125}I was purchased from Amersham-Pharmacia (Piscataway, NJ; Les Ulis, France). Oleic acid, bovine serum albumin (A2153)(BSA), were obtained from Sigma (St. Louis, MO; St. Quentin Fallavier, France). Sodium heparin was purchased from Choay laboratories (Gentilly, France). Fugene was purchased from Roche Boehringer Mannheim (Indianapolis, IN), and Superfect from Qiagen (Valencia, CA). Zeocin was obtained from Invitrogen (Carlsbad, CA). Suramin was a gift from Bayer Pharmaceuticals (Puteaux, France). Enzymatic kits for the determination of TG and FFA were obtained from Roche-Boehringer Mannheim (Meylan, France) and WAKO (Richmond, VA; Unipath, Dardilly, France), respectively. Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin-streptomycin, glutamine, and fetal bovine serum (FBS) were purchased from Life Technologies, Inc (Grand Island, NY; Eragny, France). RIA kits for plasma leptin measurements were obtained from Linco (St. Louis, MO). Experiments in Figures 1 (*db/db* only), 2 and 6 were performed using recombinant mouse leptin prepared in the laboratory as described previously (Yen F.T., Masson M., Clossais-Besnard N.,

Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398).

The remainder of the experiments were performed using commercial preparations of recombinant human or mouse leptin (Sigma and Calbiochem, Meudon, France). α_2-Macroglobulin-methylamine was a kind gift from Dr. D. Strickland (American Red Cross, Rockville, MD).

Animals

Male wild-type and C57BL/Ks *db/db* (db) mice were purchased from R. Janvier Breeding Center (Le Genest St. Isle, France), while male db^{Pas}/db^{Pas} were kindly made available by Prof. J.L. Guenet (Institut Pasteur, Paris, France). Female *ob/ob* mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an animal facility on a 12 h light/dark cycle and were allowed water and rodent chow (No. 113, UAR, Epinay-sur-Orge, France) *ad libitum*. Mean body weights at the time of the experiment for wild-type, *db/db*, db^{Pas}/db^{Pas}, and *ob/ob* mice were 27.8 ± 1.4, 33.8 ± 9, 74.6 ± 11.4 g, and 49.4 ± 5.49 g, respectively. The research protocol was in accordance with French Ministry of Agriculture, section of Health and Animal Protection and the established institutional guidelines.

Cells

Primary cultures of rat hepatocytes were prepared as described previously (Yen, F.T., Mann, C.J., Guermani, L.M., Hannouche, N.F., Hubert, N., Hornick, C.A., Bordeau, V.N., Agnani, G., and Bihain, B.E. (1994). Biochemistry *33*, 1172-1180). using overnight-fasted 150-200 g Sprague-Dawley male rats (R. Janvier Breeding Center) or obtained commercially (InVitro Technologies, Baltimore, MD). Cells were used in experiments 48 h after plating. The PLC liver hepatoma (CRL-8024) and Chinese hamster ovary (CHO-K1, CRL 9618) cell lines were obtained from the ATCC repository (CRL-8024; Manasass, VA). The PLC line was maintained in tissue culture with MEM containing 10% (v/v) FBS, 2 mM glutamine, sodium pyruvate, non-essential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin. CHO-K1 cells were grown in Ham's-F12 containing 10% (v/v) FBS, 2 mM glutamine and 100 units/mL each of penicillin and streptomycin.

Anti-LSR antibodies and peptides

The preparation of antibodies directed against rat LSR protein, and anti-LSR peptide 170 antibodies was as described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398. Synthetic peptides 81B and 93A with sequences corresponding to human LSR α residues 35-45 (FGRDARARRAQ) and 613-627 (EEAYYPPAPPPYSET),

respectively, were obtained commercially. Polyclonal antibodies directed against this synthetic peptide conjugated to KLH were prepared, and the IgG fraction was purified as described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L. Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398.) Synthetic peptides corresponding to residues 117-138 of mouse leptin (CSLPQTSGLQKPESLDGVLEAS) as well as the corresponding fragment of human leptin were commercially prepared (Research Genetics, Huntsville, AL).

In Vivo Methods

Measurement of plasma lipid response in mice

Mice that were fasted for 2-3 hours were gavage-fed 300 µL of a test meal consisting of 60% fat (37% saturated, 27% mono-, and 36% polyunsaturated fatty acids), 20% protein and 20% carbohydrate, and providing 56 kcal of energy/kg (1.5 g butter, 1.5 g sunflower oil, 2.5 g nonfat dry milk, 2.5 g sucrose and 3 ml water). Immediately after the meal, the animals were injected intravenously (*db/db*) or intraperitoneally (db^{Pas}/db^{Pas}) with either 200 µL physiological saline or 200 µL of the same solution containing recombinant mouse leptin. At selected time intervals, 20 µL of blood were collected from the orbital (db^{Pas}/db^{Pas}) or tail (*db/db*) vein into ice-cold microfuge tubes containing 4 mmol/L EDTA. Plasma was obtained by centrifugation at 2500 rpm for 20 min at 4 °C, and was frozen as aliquots at –80 °C before analysis. TG concentrations were determined using a commercially available enzymatic kit with controls included in each assay (Precinorm L, Roche-Boehringer Mannheim; Lyotrol N, BioMérieux).

Measurement of postheparin lipolytic activity

Mice were gavage-fed and injected with leptin or control solutions as described above. At t = 1 h, the mice were injected subcutaneously with heparin (100 IU/kg body weight). At t = 2 h, the animals were bled and the plasma was immediately separated by centrifugation. Lipase activity was determined according to Iverius and Brunzell (1985) using 20% Lipoven (Fresenius France Pharma, Louviers, France) as the source of TG. The assay was performed using 25 µL postheparin plasma in 0.15 M NaCl (200 µL total volume), and in the presence of 10 µL heat-inactivated (56 °C, 30 min) human plasma as a source of apoC's. Before and at the end of the incubation, FFA concentrations were determined using an enzymatic kit.

Cell Culture Studies

Lipoprotein receptor studies

LSR activity was measured as the oleate-induced binding, uptake, and degradation of ^{125}I-low density lipoprotein (LDL) in cells following the method described in detail previously (,

B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low-density lipoproteins in fibroblasts from a subject homozygous for familial hypercholesterolemia. Biochemistry *31*, 4628-4636; Yen, F.T., Mann, C.J., Guermani, L.M. Hannouche, N.F., Hubert, N., Hornick, C.A., Bordeau, V.N., Agnani, G., and Bihain, B.E (1994). Biochemistry *33*, 1172-1180) ; Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398). Modifications of the standard protocols are described in the Brief Description of the Drawings.

Identification Of LSR Protein

Western blotting

Confluent monolayers of cells were washed in PBS, and lysed in 20 mM Tris containing 2 mM EDTA and 0.5 % (w/v) SDS and an protease inhibitors (0.1 mg/mL PMSF, 2 µg/mL leupeptin and 1.9 µg/mL aprotinin). The lysate was then separated on 10% SDS-PAGE under denaturing conditions. After transfer to nitrocellulose, the strips were probed with anti-LSR peptide anti-serum. Bands were revealed after incubations with secondary goat anti-rabbit IgG conjugated to alkaline phosphatase. After washing in PBS containing 0.5% (v/v) Tween 20, the bands were revealed by incubation with substrate.

Immunoprecipitation

Confluent monolayers of PLC cells were lysed in PBS containing 1% (w/v) Triton X-100, and then were incubated with the specified anti-LSR antibodies, as described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398). Immunoprecipitates were separated on 10% SDS-polyacrylamide gels under nondenaturing conditions, and then transferred onto nitrocellulose.

Ligand blotting

Partially purified rat LSR (240 kDa band complex) was obtained as described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398.) The band was separated on non-denaturing 4-12% gradient SDS polyacrylamide gel, and was transferred to nitrocellulose by semi-dry transfer (Biorad, 18 V, 25 min). The nitrocellulose strip was incubated at room temperature with PBS containing 3% BSA, and then incubated at 37 °C for 1 h with 200 ng/mL 125I-leptin in PBS containing 0.2% BSA, pH 7.4. After six 10 min washes in PBS containing 0.5% TritonX-100, the strip was air-dried and exposed on a phospohor screen for analysis.

Preparation of lipoproteins

Human LDL ($1.025 < d < 1.055$ g/mL) were isolated by sequential ultracentrifugation of fresh plasma obtained from the local blood bank (Havel, R., and Kane, J.P. (1995). In The Metabolic and Molecular Basis of Inherited Disease, vol. II, Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds. (New York, NY: McGraw-Hill, Inc), pp. 1841-1851.

Rat chylomicrons were prepared from overnight-fasted male Sprague-Dawley rats (300-400 g) given a high-fat liquid meal similar to that given to mice (2 mL per animal). After 45 min, the animals were anesthetized and catheters were inserted in the main abdominal lymph duct. Lymph was collected over 2 hours, and the chylomicrons were isolated. Contaminating albumin was removed by incubation for 30 min at room temperature with an equivalent volume of swollen Blue Sepharose CL-6B gel (Amersham Pharmacia Biotech) (Mann, C.J., Troussard, A.A., Yen, F.T., Hannouche, N., Najib, J., Fruchart, J.-C., Lotteau, V., André, P., and Bihain, B.E. (1997). J. Biol. Chem. *272*, 31348-31354). All lipoproteins were stored in the dark at 4 °C under N_2 and used within 2 weeks (LDL) or 3 days (chylomicrons) of their isolation

Radiolabelling

Lipoproteins were radioiodinated using Bilheimer's modification of the McFarlane's procedure (Bilheimer, D.W., et al. (1972). Biochim. Biophys. Acta 260, 212-221), and used no more than 1 week after radiolabeling. ^{125}I-LDL was filtered (0.2 μm, Gelman, Ann Arbor, MI) on the day of the experiment.

Leptin was iodinated using Iodobeads (Pierce) according to the manufacturer's instructions.

Cloning Of Full Length cDNA Human LSR

Human homologous sequences of rat LSR cDNA were found with 2 partially overlapping human genomic sequences (Genbank accession nos: AD000684 and AC002128). ESTs generated on the basis of these sequences were used to screen a human BAC library. A single clone was isolated and sequenced. Analysis of this sequence revealed several variations from the public sequence. A revised LSR sequence is currently available in Genbank (accession numbers TBA).

An 805 bp fragment was obtained by PCR amplification of human liver mRNA (Sense primer: 5'- CTACAACCCCTACGTCGAGT (SEQ ID NO:22), antisense primer: 5' – AGGCGGAGATCGCCAGTCGT (SEQ ID NO:23)), and subcloned into the TA cloning vector (Invitrogen, Carlsbad, CA). The cloned insert was isolated by digestion with EcoR1, was purified (GenClean kit, Bio 101, Vista, CA), and the DNA was labeled with α-^{33}P-dCTP (NEN, Boston, MA) using the random primers labeling system (Life Technologies). The labelled fragment was used to screen the cDNA library (Superscript, Life Technologies), from which we obtained a partial α' clone (clone 18251), lacking 161 bp of the 5' region.

The missing 5' region was obtained by PCR amplification (AmpliTaq, Promega, Madison, WI) from a first strand cDNA prepared from human liver total RNA (Clonetech, Palo Alto, CA)(both oligo dT and random primers were used). The primers for PCR were sense 5'CCTTTGTCCACGTCGTTTACGCTC-3' (SEQ ID NO:24) and antisense 5'-TCACAGCGTTGCCCTGCTTG –3' (SEQ ID NO:25). The PCR was performed with annealing temperature of 65 °C and 35 cycles. The fragment was cloned into pGEMT-Easy Vector (Promega).

Fragments corresponding to the α forms and β were cloned into pGEMT-Easy Vector and then used to replace the appropriate region in the LSR α' clone. The full-length LSR α, α', and β clones were reconstructed in pTracer-CMV2 vector (Invitrogen) using EcoRI/Xba I.

PCR Analysis of Human LSR

Similarly to previous results with rat LSR (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398), two splice variants of LSR were detected by RT-PCR analysis of human hepatocyte cDNA. In Fig. 3B, sense and antisense primers were designed to yield three products, of which two were the splice variants. The primer sequences were: sense, 5'-TTACTGCTCCGTGGTCTCAGC- 3' (SEQ ID NO:26) and antisense, 5' AGCTACTCCTGTCAACGTCTCC – 3' (SEQ ID NO:27). Identities of each band were confirmed by sequencing.

Northern Blotting

Northern blots were performed as described previously using as a probe clone 18251 described above (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398).

In vitro translation

In vitro translation products were obtained using ^{35}S-methionine (Amersham) and the T7 coupled transcription/translation kit from Promega.

Transient Transfection Studies

CHO-K1 cells were plated at a density of 300,000 cells/36 mm dish the day before transfection. After 24 h, plasmid preincubated with Fugene transfection reagent was added to the cells, which were further incubated at 37 °C. Cells were used 48 h after transfection as described in the Brief Descriptioon of the Figures.

Stable Transfections

Stable transfectants were prepared from CHO-K1 cells using Superfect according to the manufacturer's instructions. After introduction of the plasmid into the cell with Superfect, the cells were grown in the presence of 750 μg/mL zeocin. After elimination of untransfected cells, the antibiotic concentration was reduced to 500 μg/mL. Clones were isolated using cloning cylinders, and maintained in tissue culture media containing 100 μg/mL zeocin.

FACS Analysis

Flow cytometry is a laser-based technology that is used to measure characteristics of biological particles. The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as light from the excitation source strikes the moving particles.

Assay 1 :PLC cell suspensions were obtained using non-enzymatic dissociation solution (Sigma), and then were incubated for 1 h at 4 °C with a 1:200 dilution of anti-LSR 81B or irrelevant anti-serum in PBS containing 1% (w/v) BSA. After washing twice with the same buffer, goat anti-rabbit FITC-conjugated antibody (Rockland, Gilbertsville, PA) was added to the cells, followed by a further incubation for 30 min at 4 °C. After washing, the cells were fixed in 2% formalin. Flow cytometry analysis was done on a FACSCalibur cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Assay 2 : Cells are cultured in a T175 flasks according to manufacturer's instructions for 48 hours prior to analysis.

Cells are washed once with FACs buffer (1x PBS/2% FBS, filter sterilized), and manually scraped from the flask in 10 mLs of FACs buffer. The cell suspension is transferred to a 15 mL conical tube and centrifuged at 1200 rpm, 4 °C for 5 minutes. Supernatant is discarded and cells are resuspended in 10 mL FACs buffer chilled to 4 °C. A cell count is performed and the cell density adjusted with FACs buffer to a concentration of 1 $x10^6$ cells/ mL. One milliliter of cell suspension was added to each well of a 48 well plate for analysis. Cells are centrifuged at 1200 rpm for 5 minutes at 4 °C. Plates are checked to ensure that cells are pelleted, the supernatant is removed and cells resuspended by running plate over a vortex mixer. One milliliter of FACs buffer is added to each well, followed by centrifugation at 1200 rpm for 5 minutes at 4 °C. This described cell washing was performed a total of 3 times.

Primary antibody, titered in screening experiments to determine proper working dilutions (for example 1:25, 1:50, 1:100, 1:200, 1:400, 1:500, 1:800, 1:1000, 1:2000, 1:4000, 1:5000, or 1:10000), is added to cells in a total volume of 50 μL FACs buffer. Plates are incubated for 1h at 4 °C protected from light. Following incubation, cells are washed 3 times as directed above. Appropriate secondary antibody, titered in screening experiments to determine proper working dilutions (for example 1:25, 1:50, 1:100, 1:200, 1:400, 1:500, 1:800, 1:1000, 1:2000, 1:4000, 1:5000, or 1:10000), is added to cells in a

total volume of 50 µL FACs buffer. Plates are incubated for 1h at 4 °C protected from light. Following incubation, cells are washed 3 times as directed above. Upon final wash, cells are resuspended in 500 µL FACs buffer and transfered to a FACs acquisition tube. Samples are placed on ice protected from light and analyzde within 1 hour.

Protein Determinations

Protein concentrations were determined using Markwell's modified Lowry procedure (1981) or BCA protein assay (Pierce Chemical Co, Rockford, IL) and BSA as standard.

Statistical Analysis

Results were analyzed using unpaired Student's t-test.

EXAMPLE 1: Effect of Leptin on Postprandial Plasma TG Response

Transient hypertriglyceridemia seen after administration of a test meal in two strains of obese mice with defects of the Ob-Receptor (OB-R) is shown in Figures 1A and 1B (open symbols). The *db/db* mice present a mutation of the Ob-Rb isoform, preventing signaling to the JAK and Stat system, while the db^{Pas}/db^{Pas} lack any leptin signaling capacity through the Ob-R. Similar to what is observed in most obese human subjects (Lewis, G.F., O'Meara, N.M., Soltys, P.A., Blackman, J.D., Iverius, P.H., Druetzler, A.F., Getz, G.S., and Polonsky, K.S. (1990) *J. Clin. Endocrinol. Metab.* **71**, 1041-105o ; Vansant, G., Mertens, A., and Muls, E. (1999) *Intl. J. Obesity* **23**, 14-21) postprandial plasma lipid levels were elevated in both strains of obese mice when compared to lean controls (shown as dotted lines). A single bolus injection of 50 µg leptin at the time of the meal decreased the amplitude of the triglyceride response (Fig. 1A and 1B, closed symbols); this effect could not be attributed to a reduction in food intake since the meal was administered by intragastric cannulation.

A significant reduction of the area under the TG curve was observed with 250 ng of leptin per animal (Fig. 1A, 1B, insets). It can be estimated (average body weight of db^{Pas}/db^{Pas}, 74.6 ± 11.4 g; plasma volume 45 mL per kg) that this dose cannot cause more than a two-fold increase of the concentration of circulating leptin (86.7 ± 12.2 ng/mL) in db^{Pas}/db^{Pas}. Maximum effect of leptin was achieved with 500 ng per animal which decreased by > 80% and > 65% the area under the postprandial TG curve in *db/db* and db^{Pas}/db^{Pas}, respectively. This dose of leptin (7 µg per kg body weight) is 15-fold lower than that used to achieve 30 to 40% reduction of food intake after peripheral administration of leptin (Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995) *Science* **269**, 546-549; Halaas et al, 1995; Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and, J.M. (1995) *Science* **269**, 543-546; Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) *Science* **269**, 540-543). These

data establish that leptin can control the exogenous lipoprotein pathway and that this regulation occurs in db^{Pas}/db^{Pas} in spite of the complete defect of the Ob-R.

EXAMPLE 2: Leptin Binding to Rat LSR

The binding of leptin to LSR was tested using partially purified rat LSR multimeric complexes. Complexes separated by SDS electrophoresis (Fig. 2, lane 1) and transferred to nitrocellulose, bound ^{125}I-leptin (Fig. 2, lane 3). The same bands were recognized by polyclonal anti-rat LSR antibodies (Fig. 2, lane 2). The specificity of these antibodies has been described previously (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398).

EXAMPLE 3 : Effect of LSR Subunit Transfection

To determine which of the LSR subunits is responsible for leptin binding, CHO-K1 cells were transiently transfected with increasing concentrations of each of the 3 human LSR plasmids (Fig. 3A). CHO-K1 cells were selected because they had the lowest level of endogenous LSR expression of the different cell lines tested. This level is far lower than that of a human hepatocyte cell line (PLC) used to systematically characterize human LSR activity (Fig. 3B). The data showed that only transfection with the LSR α' plasmid increased the binding of leptin to CHO-K1 cells (Fig. 3A). Leptin binding to CHO-K1 cells transfected with LSR β or α remained at levels similar to those seen with the vector alone. Analysis of the expression of co-transfected green fluorescent protein (GFP) estimated transfection efficiency at ± 25% for all 3 transiently transfected plasmids.

CHO-K1 cells stably expressing LSR α' were also obtained and were determined to have an increased ^{125}I-leptin binding and uptake (Fig. 3C). The apparent molecular mass of human LSR α' in stable CHO-K1 transfectant cells corresponded to that of the smallest LSR subunit (~ 70 kDa) in PLC cells (Fig. 3B). Lineweaver-Burk transformation of leptin binding to CHO-K1 cells expressing LSR α' yielded an estimated Kd of 1.3 nM (Fig. 3C, inset), ~ 2 fold that of the Ob-R (Kd = 0.7 nM; Tartaglia et al, 1995). Leptin binding to LSR α' led to its internalization and proteolytic degradation consistent with this leptin binding reflecting a biologically relevant process (Fig. 3D).

Similar to what is observed in cells transfected with the Ob-Ra or Ob-Rb (Uotani, S., Bjørbærk, C., Tornøe, J., and Flier, J.S. (1999). Diabetes *48*, 279-286.) the amount of ^{125}I-leptin degraded by CHO-K1 cells transfected with LSR α' represented only 16% of that bound and internalized by the cells. These rates of ^{125}I-leptin degradation are much lower than those observed with receptors mediating rapid endocytosis (Goldstein, J.L., Basu, S.K., Brown, M.S. (1983). *98*, 241-260). For instance, after 2 h incubation, the amount of ^{125}I-LDL degraded through LSR represents 4-5 times the amount bound to the cell surface (Bihain, B.E., and Yen, F.T. (1992). Although not intending to be

limited by any particular theory, the simplest explanation is that LSR α' lacks the di-leucine routing signal known to trigger rapid lysosomal delivery. The LSR α contains such a signal, consistent with previous observations that the α subunit is a critical element allowing LSR to function as lipoprotein receptor (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398).

Similar experiments are performed in the other stable cell lines expressing the subunits of LSR alone or in all combinations (see table, below). These cell lines are useful for screening small molecules or any potential agonist or antagonist for activity against either the leptin or triglyceride (or both) activity of LSR. In addition, they can be employed in receptor binding assays using FACS analysis or radiolabelled ligands to identify additional ligands of LSR.

LSR stable-transfectant Cell Lines
CHO LSR alpha
CHO LSR alpha'
CHO LSR beta
CHO LSR alpha'/beta
CHO LSR alpha/beta
CHO LSR alpha/alpha'
CHO LSR alpha/alpha'/beta

EXAMPLE 4 : Effect of 81B anti-LSR Antibody on LSR Binding and Degradation of Leptin

To test whether in nontransfected cells leptin binds to LSR, PLC cell lysates were immunoprecipitated with an antibody directed against a synthetic peptide with a sequence identical to LSR residues 35-45 (81B). Ligand blotting showed that ^{125}I-leptin binds directly to the multimeric complexes (apparent molecular masses of 200 and 230 kDa) precipitated by the 81B antibody (Fig. 4A). These complexes are of a size similar to that of rat LSR multimeric complexes (Yen F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398). Significant amounts of TCA-soluble chloroform-insoluble ^{125}I-leptin degradation products were found in the incubation media after 2 h incubation of PLC cells with increasing concentrations of ^{125}I-leptin (Fig. 4B, □). The dose response curve indicated that the process saturated for leptin concentrations ≥ 10 ng/mL (Fig. 4B). The amount of leptin degraded per mg of PLC cell protein is about half as much as that degraded by CHO-K1 LSR α'stable transfectants (Fig. 3D).

Chloroquine (50 μM) inhibited ^{125}I-leptin degradation by more than 60%, while increasing the amount of cell-associated ^{125}I-leptin (2-4 fold). This is consistent with ^{125}I-leptin degradation occurring in lysosomes after receptor-mediated endocytosis. The 81B antibody that immunoprecipitated LSR multimeric complexes had a profound inhibitory effect on leptin degradation in PLC cells (Fig. 4B, ■). This effect was maximal with 10 ng/mL of leptin and 200 μg/mL of antibody and was partially competed off by increasing leptin concentrations at 20 ng/mL. Because immunoprecipitation data revealed no interaction of the 81B antibody with the Ob-R or any other protein (Fig. 4A), the inhibitory effect of this antibody on leptin degradation indicates that in cells of liver origin, the LSR is quantitatively the primary mechanism for leptin degradation. FACS analysis confirmed that the 81B anti-LSR antibody binds to non-permeabilized PLC cells (Fig. 4D). This indicates that the amino-terminal is exposed on the cell surface.

Leptin binding to LSR does not require the presence of FFA and is inhibited by the 81B antibody directed towards the LSR sequence located near the amino terminal end Immunoinhibition studies previously showed that the cluster of charged residues found at the carboxyl terminal end most likely represents the rat LSR lipoprotein binding site (Yen, F.T., Masson M., Clossais-Besnard N., Andre P., Grosset J.M., Bougueleret L., Dumas J.B., Guerassimenko, O., and Bihain B.E. (1999). J Biol Chem *274*, 13390-13398). Accordingly, LSR was classified as a type II membrane receptor. FACS analysis using the 170 antibody, directed towards a synthetic peptide with a sequence corresponding to that of LSR's carboxyl terminal end, is consistent with this interpretation (Fig. 4D).

While not wishing to be limited by any theory, the observation that the 81B antibody inhibits leptin binding to LSR and binds to intact PLC cells (FACS analysis, Fig. 4D), suggests that LSR amino terminal ends are also exposed on the external side of the plasma membrane. LSR contains a typical 28 amino acid transmembrane spanning domain located between residues 259-286 (Fig. 4C). In addition, a cluster consisting of 3 stretches of hydrophobic amino acids is located towards the amino terminal end. Each of these hydrophobic clusters is too short to allow crossing of the plasma membrane, but since the three hydrophobic elements are in close proximity with only two short hydrophilic separating clusters, a transmembrane spanning region could be constituted. In this case, the two separating hydrophilic domains would be oriented inwardly to minimize interaction with the hydrophobic moieties of the phospholipid bilayers. According to this model, LSR α and α' could cross the plasma membrane twice, with both carboxyl and amino terminal ends protruding extracellularly. LSR β would be limited to a single crossing of the membrane.

EXAMPLE 5 : Effect of Leptin on LSR Activity

The effect of leptin on the activity of LSR with respect to its ability to bind, internalize and degrade lipoproteins was also studied. Leptin directly increased the oleate-induced LSR binding uptake

and degradation of ^{125}I-LDL in a dose-dependent manner (Fig. 5A, 5B, 5C). The effect was observed at leptin concentrations $\geq$ 10 ng/mL.

The specificity of leptin's stimulatory effect upon LSR was further established by the observation that leptin at concentrations of up to 2 μg/mL had no detectable effect on the degradation of LDL by the LDL-receptor nor on that of activated α_2-macroglobulin, the preferred LRP ligand.

The stimulatory effect of leptin on LSR activity as a lipoprotein receptor was suppressed by the 81B antibody (Fig. 5D). The antibody 170 directed against a rat LSR sequence located towards the carboxyl terminal end was used as a control. Although the 170 antibody had an inhibitory effect on the oleate-induced ^{125}I-LDL binding in human PLC incubated without leptin, it did not prevent the leptin stimulatory effect on LSR activity (Fig. 5D).

The stimulatory effect of leptin on LSR activity as lipoprotein receptor was seen not only in cells of human origin, but also in rodent hepatocytes. A brief, 30 min, preincubation of rat hepatocytes with 20 ng/mL mouse recombinant leptin at 37 °C increased oleate-induced ^{125}I-LDL binding to the cell surface in subsequent incubations at 4 °C (Fig. 6A), indicating that this stimulatory effect of leptin occurred rapidly. Northern blots showed that this leptin treatment did not increase mRNA levels significantly. Further, inhibition of cell protein synthesis (50 μM cycloheximide) did not suppress the stimulatory effect of leptin, while microfilament inhibitors (50 μM cytochalasin B) reduced leptin stimulation by more than 80%. While not wishing to be limited by any particular theory, these results are consistent with the stimulatory effect of leptin on LSR activity resulting primarily from mobilization of a cryptic pool of receptors to the cell surface.

Figure 6B shows the additive stimulatory effect of leptin and oleate on the binding of chylomicrons to rat hepatocytes. This leptin and oleate-induced binding of chylomicrons to rat hepatocytes was suppressed by specific polyclonal anti-LSR antibodies (Fig. 6C). Thus, the stimulatory effect of leptin on LSR is not limited to LDL, but extends to TG-rich lipoproteins that are directly responsible for the transport of dietary lipid. The data show that physiological amounts of leptin acutely regulate the removal of dietary TG by the liver, and that *in vitro,* the same concentrations of leptin regulate LSR activity in hepatocytes while leaving that of other lipoprotein receptors unchanged.

The inhibition of the intestinal absorption of dietary lipids by leptin was also investigated. Overnight-fasted *ob/ob* mice were gavage-fed a high fat test meal. Immediately after the test meal (time = 0 h), the mice were injected intravenously with 200 μL saline containing either no supplement, 0.5 μg recombinant mouse leptin, 2.5 mg lactoferrin, or a mixture of 0.5 μg leptin and 2.5 mg lactoferrin. Blood samples were taken at 2 and 3 h after the test meal, and plasma TG concentrations were measured (see Table, below). Values for these 2 time points were pooled and are presented as means ± SD of quadruplicate determinations obtained in 2 different animals for each condition (* $p < 0.02$ (saline versus leptin; ¶ $p < 0.01$ saline versus lactoferrin; § NS (lactoferrin versus leptin + lactoferrin)).

Table

Effect of lactoferrin and/or leptin on the plasma lipid response of ob/ob mice

	Plasma TG 2-3 hours after test meal (mg/mL)
Saline	1.04 ± 0.08
Leptin	0.79 ± 0.1 *
Lactoferrin	2.02 ± 0.26 ¶
Leptin + Lactoferrin	1.96 ± 0.42 §

The amplitude of postprandial lipemia is determined by both the rate of intestinal lipid absorption and the rate of lipid clearance. To distinguish between these two possible sites of leptin regulation, we used lactoferrin, a milk protein that inhibits the removal of dietary lipid by the liver (Huettinger ,M., Retzek, H., Eder, M. and Goldenberg, H. (1988). Clin. Biochem. *21*,87-92). As shown in the Table, injection of lactoferrin in *ob/ob* mice caused a doubling of plasma TG measured during the postprandial stage. Further, leptin caused a decrease in postprandial plasma TG when injected without lactoferrin, but was unable to achieve a significant effect in mice simultaneously treated with lactoferrin. Although not wishing to be bound by a particular theory, this suggested that most of leptin's regulatory effect was due to stimulation of dietary lipid removal by the liver. Lactoferin has been shown previously to be an inhibitor of LSR at the concentration used (Yen, F.T., Mann, C.J., Guermani, L.M., Hannouche, N.F., Hubert, N., Hornick, C.A., Bordeau, V.N., Agnani, G., and Bihain, B.E. (1994) Biochemistry *33*, 1172-1180; Mann, C. J., Khallou, J., Chevreuil, O., Troussard, A.A., Guermani, L.M., Launay K., Delplanque, B., Yen, F.T., and Bihain, B.E. (1995) *Biochemistry 34*,10421-10431).

The effect of leptin injection on the activity of lipolytic enzymes that are involved in the hydrolysis of plasma TG was also examined. Injections of leptin (50 µg/animal) did not significantly modify lipase activity released in serum of db^{Pas}/db^{Pas} after heparin injections (Fig. 12). If anything, leptin decreased, although not significantly, the lipase activity when compared to the effect of administering the test meal alone. These data ruled out the possibility that leptin regulates postprandial lipemia by directly controlling the activity of lipolytic enzymes.

EXAMPLE 6 : Comparison of the Effect of Human and Mouse Leptin

To establish a link between leptin control of postprandial lipemia in mice and its stimulation of LSR in cultured cells, the species specificity in the ability of mouse and human leptin to activate LSR in cultured cells was utilized. Mouse leptin was more efficient than human leptin in stimulating LSR-mediated LDL degradation in primary cultures of rat hepatocytes (Fig. 7A); binding and uptake of ^{125}I-LDL followed a pattern superimposable to that of ^{125}I-LDL degradation. Conversely, human leptin was more efficient in stimulating LSR activity in human PLC cells than mouse leptin (Fig. 8B).

The effect of human (1 μg/animal) and mouse (0.25 μg/animal) leptin on plasma TG response of db^{Pas}/db^{Pas} mice was also compared. The data showed that human leptin slightly reduced the postprandial plasma TG response (Fig. 7B, closed bar), but the effect did not reach statistical significance. This is consistent with the relative inability of human leptin to stimulate rodent LSR activity in cultured cells (Fig. 7A, closed bar). Mouse leptin injected at a 4-fold lower dose had a pronounced effect on postprandial plasma TG (Fig. 7B, hatched bar), consistent with its profound stimulatory effect on LSR in cultured cells (Fig. 7A, hatched bar). Thus, the effects of human and mouse leptin on postprandial TG response in obese mice paralleled their ability to stimulate LSR activity as lipoprotein receptor in cultured cells. Such species specificity has not been shown for the Ob-R.

EXAMPLE 7 : Differential Effect of Mouse and Human Leptin and Leptin Peptide in Cells

Species specificity has been observed with respect to leptin's ability to increase LSR activity in rodent or human liver cells (Fig. 8A and 8B). Mouse leptin increases LSR activity more in rat hepatocytes, and human leptin increases LSR activity more in human cells. In human cells the mouse leptin is inactive and almost approaches an inhibitory effect.

An internal seqment of the leptin polypeptide that is near the carboxy terminus was found to differ significantly in different species (See shaded area in Fig. 13). The mouse and human sequence of this segment was synthesized as a 22-mer peptide and tested for activity in cells (Fig. 9 & 10). The human peptide was agonistic for LSR activity in human cells, while the mouse peptide was antagonist for LSR activity in human cells. Thus, the human leptin peptide has a complete signalling capacity in human cells (Fig. 9). In primary cultures of rat hepatocytes, both peptides increased oleate-induced LDL binding, though not to the same exten (at concentrations < 50 μg/mL)t. However, there was an inhibitory effect on oleate-induced LDL degradation, indicating that these peptides do not completely mimic the activity of leptin in the rat system (Fig. 10).

EXAMPLE 8 : Effect of Mouse Leptin or Leptin Peptide on the Post-prandial Response

The apparent Kd of LSR for leptin is in the same range as that of the Ob-receptor, suggesting that the regulation of LSR activity by leptin could represent a physiologically relevant process. To address this issue, the variation in plasma leptin concentration that occurs after administration of a test meal to normal mice was measured. Leptin concentrations of 1.9 ± 0.7 and 4.5 ± 0.2 ng/mL ($p<0.007$, $n=4$) were measured before and 2 h after the meal. However, in normal mice, the postprandial increase in plasma TG remained small and transient, even when massive amounts of dietary lipid were provided by intragastric cannulation. This reflects the fact that in normal mice, the rate of lipid clearance is adapted to that of intestinal absorption.

Imbalance of this system appears to occur only in obese mice. However, db^{Pas}/db^{Pas} mice are not a satisfactory model to test the physiological effect of leptin. The plasma leptin levels of these animals

are extremely high (86.7 ± 12.2 ng/mL) and furthermore, do not detectably vary after administration of a test meal. Two hours after the test meal, leptin concentrations were measured as 86.6 ± 18.9 ng/mL (NS, n=5). Therefore, *ob/ob* mice that lack leptin were used to test whether administration of a physiological dose of leptin modulates postprandial lipemia.

As seen in Fig. 11A, a single subcutaneous injection of 50 ng of leptin in *ob/ob* mice decreases the postprandial lipemic response. This injection caused a transient increase in plasma leptin concentrations up to 3.25 ± 0.03 ng/mL at 2 h; baseline values were recorded 4 h after injection. The dose of leptin that is needed to control postprandial lipemia in *ob/ob* mice is 5-10 fold lower than those used in leptin-resistant *db/db* mice. In *ob/ob* mice, the signaling effect of leptin could result either from interaction with the Ob-receptor or the LSR.

A synthetic peptide with a sequence identical to that of mouse leptin between residues 117-138 was obtained and found to stimulate the oleate-induced binding of ^{125}I-LDL in primary cultures of rat hepatocytes (Fig. 11B, insert). Daily subcutaneous injections of 25 μg of this synthetic leptin peptide to *ob/ob* mice had no effect on the food intake over a 12 day period (7.6 ± 0.4 g/day in *ob/ob* receiving saline and 6.7 ± 0.3 g/day in *ob/ob* receiving peptide; n = 4, NS). Daily injections of 25 μg of mouse leptin caused a reduction of food intake to 4.7 ± 0.5 g/day (n = 3; $p < 0.003$ versus controls). Thus, the synthetic peptide that activates LSR *in vitro* does not influence food intake by activating the Ob-receptor. Injection of 50 ng of this synthetic peptide reduced the postprandial lipemic response in *ob/ob* mice (Fig. 11B).

EXAMPLE 9: Relevance to Disease States

The instant invention has shown that leptin regulates cellular functions in the absence of functional Ob-R. A myriad of peripheral regulatory effects of leptin have been identified and attributed to leptin signaling through the Ob-R, even when the targeted tissues lack the long isoform of the Ob-R, i.e., the sole isoform with a clearly established signaling capacity (Friedman, J.M., and Halaas, J.L. (1998). Nature *395*, 763-770). The characterization of a leptin receptor distinct from the Ob-R and controlling the entry of exogenous TG into the liver opens the possibility that leptin controls other aspects of cell metabolism independently of the Ob-R. Although not wishing to be limited to a particular theory, one hypothesis is that leptin resistance is due to desensitization of the signaling pathway through which leptin binding to LSR leads to mobilization of the receptor to the cell surface.

Leptin regulation of the exogenous lipoprotein pathway opens new perspectives towards the understanding of the relationship between obesity, hypertriglyceridemia and cardiovascular disease. Indeed, accumulation in plasma of the residues of chylomicrons has been shown to increase the risk of cardiovascular disease due to the formation of atherosclerotic plaque (Karpe et al, 1998 Atherosclerosis 141, 307-314). Hypertriglyceridemia is also considered an independent predictor of cardiovascular disease in obese subjects with Type II diabetes (Feeman, 1998 Ann. Intern. Med. 128, 73-74).

By increasing the contribution of the liver to the removal of plasma TG, leptin prevents deposition of dietary lipid in adipose tissue in excess of their FFA-releasing capacity. Thus the liver plays a critical but underestimated role in the pathogeny of obesity.

EXAMPLE 10: Molecular Modeling of an Active Leptin Fragment of the Invention

The amino acid sequence for the human leptin fragment with activity is: NH_2-CHLPWASGLETLDSLGGVLEAS-COOH (SEQ ID NO:57; residues 117-138). The amino acid sequence of the mouse leptin fragment with inhibitory activity in the human system is: NH_2-CSLPQTSGLQKPESLDGVLEAS-COOH (SEQ ID NO:67).

A molecular dymamic assay (MD) was performed on both the human and the mouse 22aa peptides. MDs were performed under AMBER force field, in vacuo, with a dielectric constant proportional to 4r, a switched cutoff with inner radius of 10A and outer radius of 14A, a heatıng phase of 20ps from 0 to 300K by steps of 50K, and a production phase of 120ps at 300K. At the end of the 120ps MDs, both peptides have lost their short helical part, and have shrunk to a more compact conformation.

The maın difference between the human and mouse 22aa peptides in the packed conformations is the presence of a residue with higher accessibility (namely L133, before the 2 Glycines of the end sequence LGGVLEAS) in the human 22aa peptide.

In order to decipher which amino acid is important amoung the 126-129 amino acid residues, which differ significantly between human and mouse, the following in-silico combinatorial mutational assay was performed.

Each residue in positions 126-129 of the 22aa human peptide (conformation extracted from the human leptin) was mutated, resulting in 16 mutated peptide models. Each model was minimized until reaching an rms gradient of 0.1 Kcal/mol (within the AMBER force field). Then, each minimized model was used as the starting conformation of ultra-short molecular dynamics (MD) assay (heating phase from 0K to 300K of 20ps, and production phase at 300K of 20ps, in vacuo, under the same conditions as described above). The final MD snapshots were re-minimized, and the corresponding energies are given in the following HTML table, as well as the sequence of the spontaneously formed alpha helices.

Energies of 16 Mutated Human 22aa Leptin Peptides

Central Sequence	LD	LE	PE	PD
ET	*-87.4*	-79.3	-83.9	-69.3
	LDSLGG (SEQ ID NO:42)			TPDSL (SEQ ID NO:46)
QT	-66.0	-83.3	-68.0	-65.4
	GLQTLDSLG (SEQ ID NO:47)	GGVLE (SEQ ID NO:48)		TPDSLG (SEQ ID NO:49)

	EK	-82.5	-93.1	-92.2	-92.2
		SLGGVLEAS (SEQ ID NO:50)	PESLGG (SEQID NO:51)	PDSLGG (SEQ ID NO:52	
	QK	-83.3	-85.2	*-90.2*	-84.2
		LGGVLEA (SEQ ID NO:53)			

Left column: first 2 aa residues of the mutated ETLD (SEQ ID NO:40) human motif. First line: last 2aa residues of the mutated ETLD (SEQ ID NO:40) human motif. Information available in each cell: energy of the minimized 20ps snapshot (Kcal/mol), and alpha helix sequence if present in the 20ps snapshot. Peptides containing ETLD (SEQ ID NO:40; human motif) and QKPE (SEQ ID NO:41; mouse motif) are in italic.

Under these conditions, the EKLE (SEQ ID NO:43), EKPE (SEQ ID NO:44) and EKPD (SEQ ID NO:45) containing peptides are the most favorable ones and have an alpha helix. QKPE (SEQ ID NO:41; mouse motif) and ETLD (SEQ ID NO:40; human motif) containing peptides are the next favorable conformations, with an alpha helix for ETLD (SEQ ID NO:40). Since the residue composition of each peptide is different, both composition and conformation energies form part of the comparison, and not only conformation energies.

Other peptides of the invention that can be tested in the assays described herein or other comparable assays for LSR agonistic or antagonistic activity include the following :

Table

Human Leptin Peptide Fragments

Position	Sequence	SEQUENCE ID NUMBER
117-138	CHLPWASGLETLDSLGGVLEAS	SEQ ID NO:57
122-143	ASGLETDSLGGVLEASGYSTE	SEQ ID NO:60
127-148	TLDSLGGVLEASGYSTEVVALS	SEQ ID NO:62
132-153	GGVLEASGYSTEVVALSRGQGS	SEQ ID NO:63
112-133	AFSKSCHLPWASGLETLDSLGG	SEQ ID NO:56
107-128	LLHVLAFSKSCHLPWASGLETL	SEQ ID NO:55
102-123	ENLRDLLHVLAFSKSCHLPWAS	SEQ ID NO:54
119-136	LPWASGLETLDSLGGVLE	SEQ ID NO:58
121-134	WASGLETLDSLGGV	SEQ ID NO:59
123-132	SGLETLDSLG	SEQ ID NO:61

Table

Mouse Leptin Peptide Fragments

Position	Sequence	SEQUENCE ID NUMBER
117-138	CSLPQTSGLQKPESLDGVLEAS	SEQ ID NO:67
122-143	TSGLQKPESLDGVLEASLYSTE	SEQ ID NO:70
127-148	KPESLDGVLEASLYSTEVVALS	SEQ ID NO:72
132-153	DGVLEASLYSTEVVALSRLQGS	SEQ ID NO:73
112-133	AFSKSCSLPQTSGLQKPESLDG	SEQ ID NO:66
107-128	LLHLLAFSKSCSLPQTSGLQKP	SEQ ID NO:65
102-123	ENLRDLLHLLAFSKSCSLPQTS	SEQ ID NO:64
119-136	LPQTSGLQKPESLDGVLE	SEQ ID NO:68
121-134	QTSGLQKPESLDGV	SEQ ID NO:69
123-132	SGLQKPESLD	SEQ ID NO:71

EXAMPLE 11: Inhibition of the Expression of Endogenous LSR Using Chimeraplasty

Chimeraplasty experiments to inhibit the expression of cellular LSR are designed based on publications by Cole-Strauss et al. (Science 273 :1386-1389 (1996)) and Alexeev and Yoon (Nature Biotech. 16 :1343-1346 (1998)). The following Example is exemplary only. Other sites in LSR can be targeted using the same approach to achieve either inhibition of expression, or to change base pairs to study the importance of various residues (both protein coding and within regulatory regions , intronic, or 5' or 3' to the coding region) for LSR functioning *in vitro* and *in vivo*. Similarly, chimeric oligonucleotides can be designed to modify LSR amino acids either in the coding or non-coding regions in experimental animals and for treatment of diseases in humans.

There are two ATG codons in human LSR. The second ATG corresponds to the ATGs present in mouse and rat LSR. The first ATG is used as the start site for at least some of the forms at least some of the time, since the N-terminal antibody 81B is specific for this region of the LSR protein (See other Examples). Therefore, chimeric oligonucleotides were designed for the region after the first ATG and before the second ATG, and the region after the second ATG.

The first step was to identify regions of LSR where changing a single base pair results in the creation of a stop codon. Although there are three stop codons, TAG (amber), TAA (ochre) and TGA (stop), TGA is preferred for giving a complete stop (complete inhibition of LSR expression). Two regions were identified (one after the first ATG and one after the second ATG) where changing a single base pair would result in a TGA stop codon, and chimeric oligonucleotides were designed for the appropriate sequences (Fig. 9). Chimeric oligonucleotides are designed such that they will basically

form a double-stranded sequence with two sets of 4T's at the bends and a GC-clamp (typically 5 bases in length) at one end and the mutated sequence and its wild-type complement forming the main part of the double-stranded part (typically 25 bases in length). Flanking the mutated sequence (typically 5 DNA bases) is 2'- o-methyl RNA sequence (typically 10 bases on either side).

Primers and probes were also designed for these regions for use in an allelic discrimination assay (PE Applied Biosystems, «Allelic Discrimination Using 5' Nuclease Assays» www2.perkin-elmer.com/ab/apply/dr/dra1b4.html hereby incorporated by reference herein in its entirety including any drawings, figures, or tables). The use of flourogenic probes in a 5' nuclease assay combines PCR amplification and allele detection into a single step. Hybridization probes for the endogenous and mutant forms of the allele are included in the PCR amplification reaction. The hybridization probes are cleaved by the 5' nuclease activity of Taq DNA polymerase only if the probe's target sequence is being amplified. By using a flourogenic probe, cleavage of the probe can be detected without post-PCR processing. The flourogenic probe comprises an oligonucleotide labeled with both a flourescent reporter dye (typically 5') and a quencher dye (typically 3'). In the intact probe, the proximity of the quencher reduces the flourescent signal from the reporter dye. Cleavage liberates the reporter dye allowing an increase in its flourescent activity. The essence of the technique is that it can detect single nucleotide mismatches since these interfere with the ability of Taq DNA polymerase to cleave the probe.

Probe placement is dictated by the location of the polymorphism. Generally, the polymorphic site should be near the center of the probe, since mismatches at the ends are not typically as disruptive to hybridization. A separate probe is synthesized for each allele, and each is labeled differently (FAM and TET or JOE, for example). The main criterion for probe selection is that it be long enough to hybridize at the annealing/extension temperature used in the PCR amplification. Calculation of the annealing/extension temperature is routine for those of ordinary skill in the art. Typically a probe Tm (melting temperature) of 65-67 C works well at an annealing temperature of 60-62 C. Therefore, the length of each probe is typically adjusted so that both probes have an estimated Tm of 65-67 C. In addition, there can be no G at the 5' end, since a G adjacent to the reporter dye quenches flourescence somewhat even after cleavage. The probes can be for either strand; the strand with more C's than G's generally performs better in the 5'nuclease assay.

Primers are chosen based primarily of estimated Tms as well as small amplicon size. Primers with Tms of 58-60 C (approximately 5 C below the probe Tm) generally work well at annealing/extension temperatures of 60-62 C. Generally, primers that are unstable at their 3' ends are preferred, as this seems to reduce non-specific priming. Therefore, primers with only one to two Gs and Cs within the last 5 nucleotides of the 3' end are preferred. In addition, primers should be placed as close as possible to the probe location without overlapping the probes. This generally results in amplicons of less than 100 bp, which is advantageous for PCR amplification success.

First ATG:

Chimeric oligonucleotides. DNA is in capital letters; 2'o-methyl RNA is in small letters; mutated base is underlined:

5' – ATGCAACAGGACGGACTTGGAGTAGTTTTcuacuccaagTCAGTccuguugcauGCGCGTT TCGCGC – 3' (SEQ ID NO:74)

Allelic Discrimination Assay:

Forward Primer:	TGTCCACGTCGTTTACGCTC (SEQ ID NO:75)
Reverse Primer:	TCCCACTTCCGTTCCTTGTC (SEQ ID NO:76)
Probes (endogenous/mutant):	3' – CCTACTCCAAGTC(C /A)GTCCTGTTGCATT–5' (SEQ ID NO:77)

Second ATG:

Chimeric oligonucleotides. DNA is in capital letters; 2' o-methyl RNA is in small letters; mutated base is underlined):

5' – GACCCTGCCCTGTACCTACCTACCAGATGTTTTcaucugguagGTTCAgggcagggucGCG CGTTTT - 3' (SEQ ID NO:78)

Allelic Discrimination Assay:

Forward Primer:	GTGGTGATCCTCTTCCAGCCT (SEQ ID NO:79)
Reverse Primer:	CCAGATGACGATGGGTTGC (SEQ ID NO:80)
Probes (endogenous/mutant):	5' - ACCCTGCCCTG(T/A)CCTACCAGATGAC – 3' (SEQ ID NO:81)

The chimeric oligonucleotides are also made flourescently labeled to allow tests for transfection efficiency.

Following synthesis of the chimeric oligonucleotides and the primers and probes for the allelic discrimination assay, the flourescein-labeled chimeric oligonucleotides are transfected into PLC cells using standard methodology (other Examples), and the transfection efficiency determined by flourescence. The proportion of cells that are flourescent (successful transfection) is compared with the total number of cells by techniques that are standard in the art. If the transfection efficiency is low, various parameters of the transfection methodology may be modified to increase the transfection efficiency. These parameters are well-known in the art.

Following a successful transfection of the flourescently-labeled chimeric oligonucleotides, the unlabeled chimeric oligonucleotides are transfected into PLC cells, and the cells are sorted using FACS (flourescent activated cell sorter) after labeling cells with a first anti-LSR antibody followed by a flourescently-labeled second antibody that binds the first antibody using methods standard in the art. The first antibody can be the N-terminal specific 81B antibody to sort cells for LSR expression following mutation of the site after the first ATG, but needs to be a more C-terminal specific antibody (such as the 170 antibody (to mouse carboxy terminus) or 93A (to same region of human carboxy terminus)) to sort

cells for LSR expression tested for creation of the stop codon and expression of LSR expression following mutation of the site after the second ATG.

The cells in both groups with the lower LSR expression are collected to enrich for cells with the stop codon in at least one of the copies of LSR. The cells are then cultured and checked for the presence of the stop codon mutations using allelic discrimination. An exemplary reaction set-up and procedure is as follows :

REAGENT	FINAL CONC.	(μL)
10X TaqMan Buffer A	1X	2.5
25 mM $MgCl_2$	5 mM	5
dATP	200 μM	0.5
dCTP	200 μM	0.5
dGTP	200 μM	0.5
dUTP	400 μM	0.5
AmpliTaq Gold (5 U/μL)	1 U	0.2
AmpErase UNG (1 U/μL)	0.25 U	0.25
DEPC H_2O		2.55
TOTAL VOLUME		12.5 μL

The primer concentrations can vary from 100 nM to 300 nM. Probe concentrations can vary from 50 nM to 200 nM. Template concentrations can vary from 0.1-100 ng/reaction.

STEPS

1. 50 C for 2 min.
2. 95 C for 10 min.
3. 95 C for 15 sec.
4. 58 to 65 C for one min.
5. hold at 4 C

Repeat steps 3 & 4 for 40 cycles.

Following testing, the cells are retransfected with the chimeric oligonucleotides and again sorted for LSR expression using FACS. The cells that are expressing the lowest amounts of LSR (or none) are selected, cultured to form a homogeneous population, and rechecked using allelic discrimination to identify cell clones that no longer express LSR. These cells can then be used in assays to study the role of the various LSR subunits and the interaction of compounds with particular subunits, as well as for screening for modulators of specific LSR activities (modulated by the different subunits, for example). In addition, the above-described techniques can be used on other cells, (including those in the ATCC databank and in animals or humans) to create other kinds of cells lacking LSR activity. As well as the uses as a research and compound screening tool, the technique is also useful for treatment of diseases related to obesity *in vivo*.

Chimeric oligonucleotides were also designed to specifically inhibit either the α subunit of LSR, or both the α and the α' subunits of LSR, by targeting either Exon 4 or Exon 5, specifically.

Exon 4

Chimeric oligonucleotides. DNA is in capital letters; 2' o-methyl RNA is in small letters; mutated base is underlined):

5' –TGGCTGAGCTCTTACCTGGTTTTCATTTTtgaaaaccagGTCAGagctcagccaGCGCGTT TTCGCGC - 3' (SEQ ID NO:82)

Allelic Discrimination Assay:

Forward Primer:	GAGCTCATCGTCCTTGGGAG (SEQ ID NO:83)
Reverse Primer:	AGTCTTCTATGGGCCCCGC (SEQ ID NO:84)
Probes (endogenous/mutant):	3' CACCGACTCGAGA(A/C)TGGACCAAAAGTC 5' (SEQ ID NO:85)

Exon 5

Chimeric oligonucleotides. DNA is in capital letters; 2' o-methyl RNA is in small letters; mutated base is underlined):

5' – GGTTGTGGTATGCCTGGCTGCCTTCTTTTgaaggcagccAGTCAtaccacaaccGCGCGT TTTCGCGC - 3' (SEQ ID NO:86)

Allelic Discrimination Assay:

Forward Primer:	ACGCAGAGCTCATCGTCCTT (SEQ ID NO:87)
Reverse Primer:	GATGCCCAGGAGGAGGAAGA (SEQ ID NO:88)
Probes (endogenous/mutant):	3' – CAACACCATAC(G/T)GACCGACGGAA – 5' (SEQ ID NO:89)

For both, use FAM as the dye for the endogenous nucleotide (A and G, respectively), and JOE as the dye for the changed nucleotide (C and T, respectively).

EXAMPLE 12: Use of Zinc Finger Polypeptides for LSR Modulation

A method for specifically binding DNA of choice and repressing or initiating its transcription has been described recently in WO 98/54311. The repression or initiation can be constitutive in the presence of the vector carrying the zinc finger, or it can be placed under the control of a small molecule switch, for example the TET system, where the expression of the repressor/initiator-bound zinc finger can be regulated. This is especially important in systems where complete absence of a gene at certain developmental stages, for example, is lethal, or where it's overexpression is toxic (Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Belanger F, Langelier Y Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. J Virol 1998 Mar;72(3):2289-96 hereby incorporated by reference herein in its entirety including any figures, tables, or drawings).

Zinc finger polypeptides are designed to specifically bind to LSR genomic DNA, and then are linked with the KRAB repressor to inhibit LSR expression. Sequences identified for use in making the zinc finger polypeptides are :

1936 to 1927 of SEQ ID NO :1 TAG GGG TGA GCG GCG GGG (SEQ ID NO:91)
1947 to 1936 of SEQ ID NO :1 GAG GGC TGG NNN TAG GGG TGA (SEQ ID NO:92)
1946 to 1936 of SEQ ID NO :1 AGG GCT GGG NN TAG GGG TGA (SEQ ID NO:93)
1956 to 1947 of SEQ ID NO :1 GTG GGA GCC GAG GGC TGG (SEQ ID NO:94)
1956 to 1946 of SEQ ID NO :1 GTG GGA GCC N AGG GCT GGG (SEQ ID NO:95)
2304 to 2295 of SEQ ID NO :1 GCG GCG GCC GGG TGG GAG (SEQ ID NO:96)
1778 to 1787 of SEQ ID NO :1 TTG GCC GGA GCA GAT GGG (SEQ ID NO:97)
1787 to 1798 of SEQ ID NO :1 GCA GAT GGG NN CCG GAA GGG (SEQ ID NO98):
1946 to 1934 of SEQ ID NO :1 AGG GCT GGG NNN AGG GGT GAG (SEQ ID NO:99)
1934 to 1922 of SEQ ID NO :1 AGG GGT GAG NNN CGG GGA GGG (SEQ ID NO:100)
1740 to 1749 of SEQ ID NO :1 AAG TGG GTC TCG GTT GCA (SEQ ID NO:101)

The sequences to be bound by zinc finger polypeptides are provided to Sangamo, where the actual zinc finger proteins are synthesized and are linked to the KRAB domain, a transcription repressor (Pengue G, Calabro V, Bartoli PC, Pagliuca A, Lania L Repression of transcriptional activity at a distance by the evolutionarily conserved KRAB domain present in a subfamily of zinc finger proteins. Nucleic Acids Res 1994 Aug 11;22(15):2908-14) hereby incorporated by reference herein in its entirety including any figures, tables, or drawings), are synthesized. The DNA binding domain can also be linked to transcription initiators (such as VP16 ; Proceedings of the National Academy of Sciences USA 94 :5525 (1997) hereby incorporated by reference herein in its entirety including any figures, tables, or drawings) or a small molecule switch system, that is used to turn on or off the zinc finger protein linked to the repressor or initiator. Examples of small molecule switches that are effective in cells and in animals include, the Tet system, RU486, and ecdysone.

The zinc finger proteins are delivered as plasmids suitable for transfection into cells using standard techniques (Fugene, is a method of choice). The cells used include, but are not limited to, the human cell lines HepG2, PLC, Hep3B, C3A, and 293 and the mouse cell lines taoBpRcl, BpRcl, and Hepa1-6. All cells are available from ATCC. Following transient transfection, the cells are tested for LSR expression and activity using standard techniques described in this application, that may include FACS analysis to look for LSR expression on the cell surface, quantitative PCR to look at whether the message is being made, and various binding, uptake and degradation experiments to study LSR activity.

Following a determination of which zinc finger proteins are the most effective in inhibiting LSR expression, stably tranfected cell lines are created, using the techniques described in this application. These cell lines are used to then study the activity of the subunits of LSR separately and in combination by co-transfecting them into the cells either stably or transiently, or by turning on and off endogenous

LSR genes. These cell lines are the basis of assays for agonists and antagonists of LSR generally and the subunits separately and in any combination.

The zinc finger proteins are also provided as part of a supernatant associated virus, or retroviral adenovirus (for example adeno-associated viral (AAV)). These are effective gene transfer vectors for use in cells or in animals, as well as humans. Upon receipt, the AAV supernatant is amplified using techniques well-known in the art and examples are described in Xiao et al. J. Virology 72 :2224-2232 (1998), hereby incorporated by reference herein in its entirety including any figures, tables or drawings) and can include the use of helper plasmids as described in Collaco *et al* (Gene (1999) 238:397-405, hereby incorporated by reference herein in its entirety including any figures, tables or drawings). Following amplification, the supernatant is used to infect cells or preferably mice using standard techniques in the art some examples of which are provided by Snyder *et al.* (Nature Medicine 5 :64-69 (1999) and Teramoto *et al.* J. Virol. 72 :8904-8912 (1998), both of which are hereby incorporated by reference herein in their entirety including any figures, tables, or drawings.

Following infection, the cells are tested as described above ; the mice are tested for effects on fasting and post-prandial levels of triglycerides, free fatty acids, cholesterol, leptin, glucose, insulin, and adipoQ (Acrp30, Apm1) as well as fragments thereof, for example, before and after feedings as described herein. Similarly to plasmids, contructs in AAV gene transfer vectors can be co-infected. Thus, mice or cells can be co-infected with constructs containing cDNA encoding the α, α', or β subunits either alone or in combination to study their role *in vivo* and to test the effects of agonists/antagonists on specific subunits, or subunit combinations, in animals or cells.

LSR Zinc Finger Proteins

Sangamo's Universal GeneTools technology platform enables the rational design and rapid generation of highly specific ZFP transcription factors that can selectively recognize and regulate/modulate transcription of any target gene or DNA sequence. Expression of the ZFP's as fusions to activation (herpes simplex virus VP16) or repression (Kruppel-associated box A domain / KRAB-A) domains allows transcription to be specifically up or down modulated within cells. Figure 25 contains a table with a summary of the five sets of plasmids encoding ZFPs targeted to the LSR gene. Each set contains the ZFP target sequence fused to the VP16 domain (NVF), or the KRAB-A domain (NKF). The sequences for the NVF versions of these plasmids are listed in Figure 26. These engineered ZFP's are being used for the functional analysis of LSR in both cell-based assays and in animal models.

Cell Based Assays:

To determine the effect of these engineered on LSR expression, mouse hepatocytes were transfected and assayed for LSR mRNA by Northern analysis. Hepa1-6 cells transfected with ZFP-NVF constructs, were harvested 24 and 48 hours post transfection for total RNA isolation (Qiagen RNeasy

mini kit). Standard protocols were followed for Northern gels and blotting. Blots were probed with the full length mouse LSR alpha cDNA (EcoRI fragment from pTracer clone) and G3PDH DNA (Clontech). Probes were prepared using Prime-IT II random primer labeling kit (Stratagene) and ^{32}P dCTP. Quantitation of the Northern bands was done using Gel-Pro software.

Figure 27 shows an analysis of all 5 candidate ZFPs linked to VP16. Only 2 of these plasmids, 5185 and 5186, exhibited any increase in expression, 6% and 16%, respectively, at 48 hours post-transfection. Since this increase was not very large, a more detailed analysis of these 2 ZFPs by Northern and QPCR was used to confirm the up-regulation of LSR by 5185 and 5186.

Hepa1-6 cells transfected with ZFP-NVF constructs in triplicate, were harvested 24 and 48 hours post transfection for total RNA isolation (Qiagen RNeasy mini kit). Standard protocols were followed for Northern gels and blotting. Blots were probed with the full length mouse LSR alpha cDNA (EcoRI fragment from pTracer clone) and G3PDH DNA (Clontech). Probes were prepared using Prime-IT II random primer labeling kit (Stratagene) and ^{32}P dCTP. Quantitation of the Northern bands was done using Gel-Pro software. The results show an average of 28% mRNA increase with 5186 and a 24% increase with 5185 (Fig. 28). It should be noted that there was no significant increase in LSR mRNA on either Northern at the 24-hour time point.

Since the Northern analysis is not quite as sensitive as QPCR, the transcriptional increase was confirmed using QPCR. Cells were harvested 48 hours post transfection for Total RNA isolation (Ambion RNaqueous Kit). RNA was then reverse transcribed to generate cDNA for PCR analysis. Primer and Probe sets directed toward the mouse LSR and control GAPDH sequences were used to quantitate levels of transcription in ZFP transfected cells. As shown in Figure 29, QPCR results indicate a 41% increase in LSR transcription when Hepa 1-6 cells are transfected with ZFP plasmid 5186-NVF and a 30% increase with ZFP plasmid 5185-NVF. These results indicate that both 5185 and 5186 plasmids were functioning in cells.

Binding-Uptake-Degradation (BUD) studies were used to assay the ability of these plasmids to increase the cells ability to process ^{125}I-LDL. Cultures of Hepa1-6 mouse hepatocytes were transfected with ZFP's plasmids 24 hrs after plating. Cells were transfected with 1μg plasmid/well in a 6well plate, using Lipofectamine (Gibco BRL) according to manufacturer's instructions. Forty-eight hours post transfection, Oleate-induced ^{125}I-LDL binding, uptake, and degradation was measured as described herein.

Results of the BUD studies indicate increased binding and uptake of labeled LDL when Hepa1-6 cells are transfected with ZFP's 5186-NVF and 5185-NVF when compared to control transfected cells. The data in figure 30 have been corrected either for total protein (30A-30C) or for β-gal (30D-30F), which is a crude measure of the transfection efficiency. BUD data supports a role for ZFP 5186-NVF and 5185-NVF in the transcriptional activation of LSR and confirms a corresponding increase in functional activity.

The increase in LDL binding and uptake suggests an increase in expression of LSR at the cell surface. To prove this, cells transfected with the ZFPs were analyzed by Flow cytometry (FACs) Analysis. FACs analysis (described above) allows for direct estimation of the proportion of positive cells in a population, as well as an indirect measure of the level of receptor on the cell surface (mean fluorescence intensity).

Hepa1-6 cells were transfected with ZFP-NVF constructs 5186 and 5185, along with control plasmids. Forty-eight hours post transfection, cells were analyzed for cell surface expression of LSR in the presence/absence of Leptin (20 ng/mL). Staining of Hepa1-6 cells involved incubation with primary antibodies, generated in rabbits against mouse LSR NH2 terminal sequence CPDRASAIQ, or mouse COOH terminal sequence EEGHYPPAPPYSET, followed by detection with a fluorescent-labeled secondary antibody against IgG rabbit (Sigma).

Results indicate that in the presence of Leptin, Hepa1-6 transfected with plasmid 5185-NVF had a 50% increase in the level of LSR on the cell surface when compared to controls. While cells transfected with 5186-NVF had a 35% increase in LSR at the cell surface. These findings support a functional role for ZFP 5185-NVF and 5186-NVF in the transcriptional up-regulation of LSR and concomitant increase of LSR on the cell surface.

Analogous experiments are used to assess the efficacy of ZFP-NKFs for repressing LSR transcription.

EXAMPLE 13: Retroviral Library Screening by FACS

In order to identify more genes involved in the regulation of LSR and in ligand signaling through LSR (leptin, C1q, AdipoQ (Acrp30, Apm1), triglyceride-rich lipoproteins, etc) a retroviral library screening assay has been designed. In its most basic form, cells expressing LSR (PLC or HepG2, for example) are transfected with a retroviral library. Following sorting for expression of a marker protein, the cells are treated with a LSR ligand (leptin, for example) and assayed for LSR expression by FACS following staining with an antibody to LSR. Cells of interest, are those that either express more LSR or less LSR than is expressed following leptin stimulation of the same cells without the retroviral library.

The assay takes advantage of a retroviral vector developed by Lodish at the Whitehead Institute for Biomedical Research that takes advantage of the spectrum of expression levels of cloned cDNAs while simultaneously maintaining the high efficiency of retroviral gene transfer. The vectors employ an encephalomyocarditis virus IRES (Jang *et al*J. Virol. 62 :2636-2643 (1988)), followed by a quatitative selection marker, such as green fluorescent protein (GFP) or a cell surface marker protein, that are detectable by intrinsic fluorescence or by staining live cells with a fluorescent antibody, respectively (Fig. 14). Because expression of the two reading frames is strongly correlated , FACS sorting based on the GFP or cell surface marker protein can be used to sort the cells for those cells expressing the

unknown protein at a desired level-high, low, or moderate. For the proposed assay, the cells would preferentially be sorted for moderate expression, to allow a detectable, but not overwhelming effect.

The individual members of the gene library are placed upstream of the IRES (Fig. 14). Genes of interest for screening for their effect on LSR expression on the cell surface include cDNA libraries from liver or adipose cells. Cells expressing LSR (such as Hep3B, HepG2, PLC) would be transfected by the library using standard techniques so as to achieve approximately 1 clone (gene) per cell. The cells would then be screened, and those with moderate expression of GFP would be selected for. Cells where endogenous LSR expression has been knocked out either by traditional methods, or using the Sangamo (zinc finger proteins) or chimeraplasty techniques described herein could also be used by co-transfecting various subunits of LSR (from 1-3 and any combination thereof), or in cells stably expressing recombinant LSR subunits, or combinations.

In the GLUT 4 system, described by Lodish (Whitehead), the GLUT4 gene was linked to 7 c-*myc* epitope tags and then GFP fused in frame at the carboxy terminus. This allows the quantity of the gene to be studied in the cell compartment where it is sequestered by comparing overall fluorescence with the GFP to cell surface fluorescence with anti-*myc* antibodies. A similar assay is envisioned for LSR where LSR could be fused to GFP (in this case the library would have to be linked to CD2 or CD4). Alternatively, the amount of LSR sequestered in a cellular compartment could be determined using the 81B antibody, for example, and the amount of LSR on the cell surface could be determined using the 93A antibody, for example.

Once infected cells expressing moderate amounts of GFP are obtained, the cells can be treated with leptin, for example, (or any other LSR ligand of interest) and the difference in LSR levels in the compartment versus the cell surface, or simply on the cell surface can be determined by FACS (after antibody staining). Populations that have decreased LSR or increased LSR levels could be selected for. Optionally, the cells could be re-selected and then the retroviral DNA from the cells PCR'd and sequenced. Samples that appeared to be interesting by homologies or locations, for example, could then be cloned and re-transfected for further study. This would allow the other genes that interact with this system to be discovered. The genes are likely to encode proteins whose modulation could have a direct impact on the regulation of obesity.

EXAMPLE 14: Effect of the Leptin Peptide in Mice with Congenital Lipodystrophy

Congenital generalized lipodystrophy (CGL) is a rare autosomal recessive disorder characterized by a paucity of adipose tissue which is evident at birth and is accompanied by a severe resistance to insulin, leading to hyperinsulinemia, hyperglycaemia, and enlarged fatty liver (Seip *et al* Acta Pediatr Supp. 413 :2-28 (1996)). Leptin has been shown to reverse insulin resistance and diabetes mellitus in mice with congenital lipodystrophy (Shimomura *et al.*Nature 401 :73-76 (1999) hereby incorporated by reference herein in its entirety including any figures, tables, or drawings). These mice have extremely

low levels of leptin in plasma. However, the authors do not link the effect of leptin with LSR. The instant invention includes the use of the the leptin peptides of the invention for treatment of lipodystrophy and for use in this mouse model.

Leptin peptide will be provided to transgenic mice expressing SREBP-lc436 in adipose tissue under the control of the adipocyte-specific aP2 promoter/enhancer (Shimomura *et al.* Genes Dev. 12 :3182-3194 (1998)). The levels used are similar to those described for the *ob/ob* mice herein, a range around 50 ng per mouse. Leptin is provided daily for 12 days, either by injection, or using micro-osmotic pumps. Plasma glucose will be measured using a glucose (Trinder)-100 kit, plasma insulin by an anti-rat insulin radioimmunoassay (linco), and plasma leptin and triglyceride by standard methods described previously. A similar experiment is performed where the food intake is restricted to a level that is consumed completely by all animals.

Example 15 : Effect of Truncated Human LSR on Binding, Uptake & Degradation of LDL

Truncated forms of the LSR receptor were made and tested for their ability to function as either dominant positive (*i.e.* increase the activity of the receptor) or dominant negative proteins (*i.*e. interfere with the activity of the receptor), when over-expressed in cultured cells.

Materials:

-Human LSR cDNAs α, α' and β from constructs made in pTracer CMV2.

-pcDNA/HisMax vector from Invitrogen

-Appropriate restriction enzymes, T4 DNA polymerase I and Klenow, and T4 DNA ligase.

-Standard cloning procedures from "Molecular Cloning" by Sambrook et al.

-Follow construct plan (Figure 15).

Method of Cloning & Testing.

1. Digest Human LSR plasmids with enzymes of interest under appropriate conditions. Separate the appropriate insert fragment from the vector using agarose gel electrophoresis and Qiaquick gel extraction columns. Note: For constructs 1, 2, 3, 4a, 5, and 6 pTracerCMV2 LSR α was used as the source for the insert. For construct 4b, pTracerCMV2 LSR α' was used as the source for the insert. For 4c, pTracerCMV2 LSR β was used as the source for the insert.
2. Digest the pcDNA/HisMax vector in the appropriate reading frame with the enzymes of interest. Purify using agarose gel electrophoresis and Qiaquick gel extraction columns.
3. If necessary, treat insert fragments with Klenow DNA polymerase or T4 DNA polymerase I to blunt 3' overhangs. Purify DNA from the reaction using Qiaquick PCR purification kit.
4. Ligate inserts into vector according to Sambrook *et al.* using a 3-5 M excess of insert to vector.
5. Transform plasmids into competent *E. coli* – XL1blue from Stratagene. Follow manufacturer's instructions.
6. Isolate colonies with correct plasmids by either PCR or Qiagen miniprep analysis.

7. Verify correct clones by having them sequenced to ensure that they are in the proper reading frame and that there are no amino acid changes.
8. Grow and harvest DNA from large-scale cultures using Qiagen endotoxin free maxi preps.
9. Analyze constructs by transfecting them into human cells and assaying LDL binding using the standard BUD protocol.

BUD Assay Materials:

-DNA from LSR truncated constructs at approximately 1 mg/mL.

-Lipofectamine Plus transfection reagent – Life Technologies Cat. No 10964-013

-PLC cells plated at 0.3 x 10^6 cells/well in a 6 well plate.

- ^{125}I-LDL

-10 mM suramin (70 mL PBS per 1 g suramin)

-100 mM oleate in isopropanol, freshly prepared from a 400 mM stock solution

-DMEM (without $CaCl_2$) containing 0.2% (w/v) BSA, 5 mM Hepes, 2 mM $CaCl_2$, pH 7.5, and 3.7 g/L $NaHCO_3$ (this media should be prepared before the experiment, stored at 4°C, and used for up to 1 week)

- PBS, pH 7.4

- PBS containing 0.2 % (w/v) BSA

- 0.1 N NaOH containing 0.24 mM EDTA

BUD Assay Methods:

1. Cells (adherent) in 6-well plates seeded at 3 x 10^5 cells 3 days prior to the BUD.
   • Transfect the cells using lipofectamine plus reagent, according to the manufacturer's instructions, the day after the cells are seeded. Confluence should be between 50-80% when transfected.
   • Let cells go about 48 hrs (2 days) after transfection before BUD analysis.
2. Wash cells once with PBS (room temperature), 2 mL/well
3. Add DMEM/0.2% BSA (950 µL)
4. Add oleate, (0 to 1 mM oleate, *e.g.* 0, 0.1 mM 0.2 mM, 0.5 mM, 0.8 mM and 1 mL, from 100 mM stock)
   • never exceed 10 µL isopropanol per mL DMEM
   • It is necessary to include wells with no oleate as a control for background. This control allows one to calculate the amount of oleate-induced ^{125}I-LDL metabolized.
5. Add appropriate concentration of ^{125}I-LDL to each well (50 µL of each dilution).
6. Incubate cells for 90 min to 4 hours at 37 °C in a CO_2 incubator. In these experiments, 3 hrs was the incubation time.
7. Transfer media from wells into 5 mL polycarbonate tubes. Store at 4 °C overnight for degradation analysis (see below).

8. Wash cells at 4 °C (on ice):
    - Wash 2 times consecutively with ice-cold PBS/0.2% BSA
    - Wash once with ice-cold PBS/0.2% BSA
    - Wash 2 times consecutively with ice-cold PBS
9. Add 1 mL/ well 10 mM suramin and incubate at 4 °C for 1 hour.
10. Remove suramin into gamma counter tubes, and count for radioactivity. This represents the amount of ^{125}I-LDL bound to the cell surface.
11. Add 0.1 N NaOH/0.24 mM EDTA (1mL/well) and incubate at room temperature for a minimum of 30 min. to lyse the cells.
12. Recover the cell lysates into gamma counter tubes and count for radioactivity. This represents the amount of ^{125}I-LDL internalized. Alternatively, the suramin step may be omitted (LSR as leptin receptor) and the cells lysed immediately after washing. This would represent the amount of cell-associated ^{125}I-LDL or ^{125}I- leptin.
13. After cell lysates have been counted, determine the protein concentration per mL so that data can be reported as ng ^{125}I-LDL bound/mg of total protein. Protein is determined using the BCA assay from Pierce according to the manufacturer's instructions. Alternatively, data can be corrected for β-Gal units by transfecting extra wells and collecting them for the β-Gal assay at the time the BUD is done. For this protocol, see β-Gal protocol, below.

Degradation of ^{125}I-LDL

1.) After leaving overnight at 4 °C, add 1 mL ice-cold 40% TCA to the pre-cooled media. Do not Vortex.
2.) Incubate 1 hour at 4 °C.
3.) Centrifuge at 3000 rpm (Beckman Allegra centrifuge), 30 min @ 4 °C. (If the precipitate is floating, it is necessary to break the air-water interface by gently shaking the tubes before pelleting.
4.) Transfer 1 mL supernatant to 5 mL glass tubes.
5.) Add 40 µL 30% H_2O_2 and vortex briefly.
6.) Add 1 mL chloroform and vortex briefly. Let tubes sit for 15 minutes to allow separation of the 2 phases.
7.) Transfer 0.5 mL to gamma counter tubes, and count for radioactivity.
8.) For the calculation of the amount degraded, the dilution factor is 4.16. Corresponding plates without cells serve as controls to define the level of the background.

B-gal Assay

1). Transfect cells with test construct + 1/8th the amount of β-gal expressing plasmid.

2). Harvest cells in lysis buffer (250 µL/well of a 6 well plate). Pull through a syringe several times before transferring into an eppendorf tube.

3). Freeze cells at –80 °C until ready to perform the assay.

4). Thaw cells of interest and spin at 14K in a microfuge at 4 °C for 5 min.

5). Transfer 10 µL of each lysate to a clear PP 96 well plate:

Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank 10 µL	Blank 10 µL	empty	Sample 2 10 µL	Sample 2 10 µL	Sample 2 10 µL	Etc …					
B	Control *100 µL	Control *100 µL	empty	Sample 3 10 µL	Sample 3 10 µL	Sample 3 10 µL						
C	Sample 1 10 µL	Sample 1 10 µL	Sample 1 10 µL	Sample 4 10 µL	Sample 4 10 µL	Sample 4 10 µL						

* Control = reference standard #Blank = reaction buffer only

6). Add Fluo-Reporter β-gal substrate (Molecular Probes cat #F-2905) to β -gal reaction buffer. (275 µL CUG substrate [component A] to9.73 mL of reaction buffer) NOTE: need 10 mL for a 96 well plate, but if you don't use it all it can be stored at –20 °C for at least 6 months.

7). Add 100 µL of Reaction buffer with substrate to each well.

8). Incubate at room temp. for 30 min.

9). Add 50 µL of stop mix (0.2 M Na_2CO_3)

10). Read on Cytoflour plate reader with excitation at 360 and emission at 460. Gain should be set around 30.

β-gal reaction buffer: [Final]

0.5 M NaPhosphate pH 7.3	40 mL	0.1M
1 M $MgCl_2$	0.2 mL	1 mM
14.3 M β-mercaptoethanol	629 µL	45 mM
ddH_2O	159.171 mL	200 mL

Lysis Buffer: [Final]

Buffer II	9.875 mL	
100 % TritonX100	100 µL	1%
400 mM DTT	25 µL	1 mM

Buffer II [Final]

1 M Tris-Ac pH 7.8	50 mL	100 mM
1 M MgAc	5 mL	10 mM
0.5 M EDTA	1 mL	1 mM

ddH_2O	439 mL	500 mL

Results of BUD Assay:

Addition of the C-terminal portion of LSR increased ^{125}I-LDL binding (a), uptake (b) and degradation (c) in PLC cells (Fig. 16). ^{125}I-LDL degradation is increased almost 2 fold at 0.5 mM oleate. Data in this experiment is corrected for protein only. The transfection efficiency was not monitored. All points were done in triplicate. In a separate experiment, addition of the C-terminal portion of LSR also increased ^{125}I-LDL binding (a), uptake (b) and degradation (c) in PLC cells (Fig. 17). ^{125}I-LDL degradation was increased 2-3 fold at 0.5mM oleate. Data in this experiment was corrected for transfection efficiency only. All points are in triplicate.

The C-terminal portion of LSR from AA353 to 650 (the last AA) as well as the C-terminal portion from AA 353 to 541 are able to increase the binding, uptake and degradation of ^{125}I labelled LDL *in vitro* (Figures 16 & 17). The increase is on the order of 2-3 fold for all 3 measurements when corrected for transfection effeciency using the β-Gal reporter as a carrier in the test DNA. The increase in LDL metabolism is still on the order of 2 fold when data are corrected for total protein, depending on the oleate concentration. These constructs can be cloned into a vector to allow expression and testing *in vivo* for this dominant positive effect in animals using methods well known to those in the art.

Example 16 : LSR Gene Expression in Liver and Brain of Lean and Obese Mice

LSR gene expression was determined by quantitative PCR (QPCR) in liver and brain tissue of 7 different mouse models: normal and high fat diet-fed C57BL/6J mice (C57), C57BL6/J ob/ob (ob/ob), C57BLK/S, C57BLK/S db/db (db/db), NZB and NZO mice. The normal diet was obtained from Harlan Teklad (Teklad Certified LM-485 mouse/rat 7011C), the high fat diet, also called cafeteria diet was from Research Diets (D12331, Rat Diet 58 kcal % fat and sucrose). The cause of obesity in the different models is high fat diet in the obese C57 mice, leptin deficiency in ob/ob mice, deficiency in functional leptin receptor in db/db mice. The cause of obesity in the NZO mouse is currently unknown (Lit 1-3). C57BLK/S and NZB mice are both lean and were used as controls since they represent the corresponding background strain of db/db and NZO mice, respectively.

The qPCR results for the different LSR levels in the livers of different mouse strains are supported by immunohistochemistry result using methods well-known to persons of ordinary skill in the art.

Reverse Transcriptase – Polymerase Chain Reaction

Liver and whole brain were isolated from mice following perfusion with ice-cold saline containing 10 mM EDTA. Tissues were stored in RNAlater (Ambion, Austin) at 4 °C for 1 day and then at –20 °C. Liver total RNA was isolated using RNAqueous (Ambion, Austin) following the manufacturer's protocol. The amount of RNA was determined by absorption at 260 nm. The quality of

the isolated RNA was verified by the ratio 260/280nm (between 1.9 and 2.1 is good) and by denaturing agarose gel electrophoresis.

RNA was reverse transcribed to cDNA using oligo dT plus an LSR specific primer and Superscript II (Gibco BRL) according to manufacturer's instructions. The LSR specific primer is in exon 6 of the LSR gene (5'ACGCATGGGAATCATGGC; SEQ ID NO:90). Plasmids containing mouse LSR-α/α'/ß sequence were obtained by cloning RT-PCR products produced from mouse liver total RNA into pGEM-T easy (Promega). The sequence of the plasmid was confirmed by cycle sequencing on a ABI Prism 377 DNA Sequencer.

Quantitative PCR was performed on a ABI Prism 7700 Sequence Detection System using TaqMan technology (PE Biosystems). TaqMan assay primers and probes were designed using Primer Express software (PE Biosystems) and were synthesized by Genset, La Jolla. Each probe was double labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) covalently linked to the 5' end of the probe and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) attached to the 3' end. Uracil-N-glycosylase technology (PE Biosystems) was used to prevent contamination with PCR product.

PCR was performed using the following reagent concentrations : 25 mM MgCl2, dNTPs at 200 µM, except for dUTP at 400 µM, 1 U of AmpliTaq Gold, 0.25 U AmpErase UNG. Primers were added at 300 nM and probes at 200 nM concentration. The forward and reverse GAPDH and LSR primers used are shown in Table 1. PCR reaction conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 1 minute at 60 °C. PCR was performed in 96 well reaction plates with optical caps and fluorescence was continuously followed for each reaction. cDNA corresponding to 15 ng of total RNA were used per PCR reaction.

Quantification of LSR expression was obtained using a standard curve of the corresponding LSR plasmid covering a concentration range between $5x10^{-6}$ and $5x10^{-10}$ M (approximately 10^6 to 10^2 copies). A standard curve of mouse (C56BL/6J) total liver RNA between 200 and 0.1 ng RNA was used to determine relative levels of GAPDH expression. Amplification plots were analyzed using SDS software (PE Biosystems).

Table 1

PCR primers and probes used to determine the expression level of mouse GAPDH and mouse LSR isoforms.

Target Gene	Forward Primer	Reverse Primer	Probe
GAPDH	AACGACCCCTTCATTGACCTC	CTTCCCATTCTCGGCCTTG	ACTCACGGCAAATTCAACGGCACAG
LSR complete	GGCAGGAGAATCACCATCACA	GATCTTGGGCTGAGACCACG	TGCTGGCCTGACCTTCGAGCAGAC
LSR alpha	GCCCTTGGAAGATTGGCTCT	ATGCTTGGCACACCTGAGGT	CCAGTGCTGTCCCCACACCTGCT

LSR alpha'	ACCAGGGCAGGAGAAT CACC	GGAGGAAGAAGAGGAG GCTTG	AGCTCATTGTCCTTGATTGGCT CTTTGTG
LSR beta	TTGTCCTTGTTTATGCTG CTGG	CAGGAGAGAGGTGGGT ATAGATGC	AGCAGCCACCTCAGGTGTGCC AA

Quantification by TaqMan technology is based on determining the threshold cycle of amplification, which was determined for each unknown sample and for the standard dilutions using 0.1 fluorescence units as a threshold (maximum fluorescence > 1.5). The amount of unknown cDNA was calculated using the corresponding standard curve. LSR expression was given as absolute copy numbers and also normalized for GAPDH expression (by dividing the determined absolute copy number by the relative level of GAPDH for each individual animal). Each determination was done in triplicate and was repeated at least once; very similar results (SD<5%) were obtained.

All data were confirmed by standard Northern analysis. 16μg total RNA was pooled from 4 mice per group and tissue and analyzed by Northern. Although this type of analysis is semi-quantitative at best and LSR isoforms can not be differentiated, relative levels of gene expression show the same trends as measured by QPCR.

Results

LSR Expression in Liver

Table 1

LSR gene expression in liver of lean and obese mice (copy numbers in 15ng total liver RNA)

		LSR-alpha	LSR-alpha'	LSR-beta	LSR (sum of isoforms)	GAPDH	LSR total
C57 normal	ave	93966	110334	18454	222754	2.8	281654
	SEM	21760	16682	2790	39779	0.4	83220
	ave	42.2%	49.5%	8.3%			
	SEM	2.5%	2.4%	0.3%			
C57 obese	ave	82814	44084	17280	144177	6.0	161206
	SEM	12274	8073	2344	22521	1.7	21161
	ave	57.4%	30.6%	12.0%			
	SEM	1.2%	1.3%	0.4%			
C57 ob/ob	ave	49898	51056	21126	122079	9.1	120026
	SEM	5928	10469	1758	15113	1.0	32474
	ave	40.9%	41.8%	17.3%			
	SEM	0.7%	4.2%	3.9%			
C57BLK/S	ave	49029	68379	41340	158749	3.9	163060
	SEM	3862	3721	2043	5903	0.4	94537
	ave	30.9%	43.1%	26.0%			
	SEM	1.3%	1.6%	1.8%			
C57BLK/S db/db	ave	30625	48504	18683	97811	9.2	79745

	SEM	1953	12021	3123	10819	1.0	26413
	ave	31.3%	49.6%	19.1%			
	SEM	1.7%	7.0%	5.4%			
NZB normal	ave	98455	387287	54079	539822	3.1	588656
	SEM	4446	13253	6740	21241	0.7	27993
	ave	18.2%	71.7%	10.0%			
	SEM	0.6%	0.8%	0.9%			
NZO obese	ave	57497	225574	23377	306448	1.8	333271
	SEM	4595	11767	1091	15948	0.3	11416
	ave	18.8%	73.6%	7.6%			
	SEM	0.9%	1.1%	0.2%			

LSR Expression in Brain of Lean and Obese mice

Table 2

LSR gene expression in brain of lean and obese mice (copy numbers in 15ng total liver RNA)

		LSR-alpha	LSR-alpha'	LSR-beta	LSR (sum of isoforms)	GAPDH	LSR total
C57 normal	ave	1192	6443	7731	15365	36.2	10653
	SEM	155	1512	443	1717	3.0	1933
	ave	7.8%	41.9%	50.3%			
	SEM	0.5%	6.0%	6.3%			
C57 obese	ave	1496	10472	7418	19387	20.8	14118
	SEM	155	1295	716	1998	5.7	805
	ave	7.7%	54.0%	38.3%			
	SEM	0.5%	1.9%	2.2%			
C57 ob/ob	ave	1293	6502	6158	13954	34.2	14034
	SEM	190	797	475	863	5.2	1939
	ave	9.3%	46.6%	44.1%			
	SEM	1.0%	3.4%	4.4%			
C57BLK/S	ave	1918	5585	6456	13958	26.7	10458
	SEM	206	354	1024	1087	5.3	980
	ave	13.7%	40.0%	46.3%			
	SEM	1.7%	2.8%	4.2%			
C57BLK/S db/db	ave	1834	5195	8189	15217	35.0	10912
	SEM	199	297	789	1117	4.5	670
	ave	12.0%	34.1%	53.8%			
	SEM	0.7%	2.0%	1.4%			
NZB normal	ave	654	1019	5463	7135	17.0	4430
	SEM	159	321	929	1051	4.7	926
	ave	9.2%	14.3%	76.6%			
	SEM	1.7%	5.1%	6.8%			
NZO obese	ave	168	320	2715	3202	13.4	1446
	SEM	112	52	37	1638	4.5	1008
	ave	5.2%	10.0%	84.8%			
	SEM	12.9%	5.6%	16.8%			

C57BL6/J, C57BLK/S, db/db, ob/ob Mice

LSR expression in the liver of obese animals is significantly lower than in lean control animals (Fig. 18). In general, the expression of LSR in brain tissue is much lower than in liver. However, unlike in liver, obesity does not cause further downregulation (Fig. 21).

No significant differences in isotype patterns were found in liver samples from the different mouse models. LSR alpha and alpha' contribute equally and account for almost all of the total LSR expression. LSR beta contributes only a small percentage (Fig. 19 and Fig. 20).

In contrast, LSR alpha' and beta are the major contributors to overall LSR expression in brain, accounting in equal proportions for about 90% of total LSR message. No significant levels of LSR alpha were seen in any of the studied models (Fig. 22 and Fig. 23).

The downregulation of LSR seems to be strongly associated with obesity independent of the cause of obesity (dietary as well as different genetic defects are the causes in the used models). One might expect that upregulation of liver LSR expression in obese individuals would be beneficial.

NZB and NZO Mice

LSR expression in liver tissue of NZB mice is 2-fold higher than in normal C57 mice. Obesity (in the NZO) again leads to strong downregulation, however, this level is still significantly higher than in other obese mice (Fig. 18). In contrast, LSR expression in the brain of NZB and even more so in brain tissue of NZO, is significantly lower than in the other 5 models (Fig. 21).

Distribution of LSR isotypes in NZB and NZO mice was very different from the previously described 5 models. The dramatic increase in liver LSR expression seen in NZB (and in NZO) mice was found to be mainly LSR alpha'. This form accounted for 80% of total LSR (Fig. 19 and Fig. 20). The complete opposite was seen in brain tissue. NZB mice have very low expression of LSR alpha and alpha' with LSR beta being the dominant isoform. This picture is even more pronounced in NZO mice. Brain LSR in these animals is almost exclusively LSR beta and some animals had virtually no alpha or alpha' expression (Fig. 22 and Fig. 23).

The fact that NZO mice respond to intracerebroventricular injection of leptin but not to peripheral injection (Halaas JL, et al., Proc.Natl.Acad.Sci. USA, 94, 8878-8883, 1997) suggests a transport defect. Since LSR alpha' has been shown to bind leptin, and since LSR alpha' levels are reduced in NZO mice, the implication is that the genetic defect in NZO mice causing obesity might be deficiency in brain LSR alpha' expression resulting in non-functioning leptin transport across the blood brain barrier. This conclusion is further supported by the discovery that some NZO mice that do not become obese have LSR alpha' expressed at significant levels in brain.

Example 17 : Effect of a Ser → Asn substitution on LSR activity in human hepatocytes

Previously, we described a frequent (allele frequency 12%) G ➔ A mutation of cDNA base pair 1088 (LSR exon 6), which results in a Ser ➔Asn mutation at amino acid position 363, presumably in the extra-cellular domain of the receptor.

In a group of 34 obese adolescent girls, this coding mutation significantly increased fasting and postprandial plasma triglyceride response to a high fat test meal. In a larger population of 154 obese adolescent girls, the same coding mutation significantly and selectively influenced fasting plasma triglyceride levels and increased 3.5 fold the risk of hypertriglyceridemia. This data suggested that LSR plays a significant role in the clearance of triglyceride-rich lipoproteins. Interestingly, even individuals heterozygous at this locus showed the effect.

An *in vitro* model was obtained after sequence analysis of LSR in 2 cell lines, PLC and HepG2, revealed that PLC cells are homozygous for the G allele, while HepG2 cells are heterozygous, having both the G and A allele.

Methods:

The oleate-induced ^{125}I-LDL binding, uptake and degradation was measured in HepG2 and PLC according to the method described previously (Bihain, B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low-density lipoproteins in fibroblasts from a subject homozygous for familial hypercholesterolemia. Biochemistry *31*, 4628-4636.). Briefly, confluent monolayers of cells were washed once in phosphate buffered saline (PBS), and then incubated 3 h at 37 °C with increasing concentrations of oleate (as indicated) and 20 μg/mL ^{125}I-LDL. At the end of the incubation, cells were placed on ice and washed twice with PBS containing 0.2% BSA, once with the same buffer, and then twice with PBS alone. The amounts of ^{125}I-LDL bound, internalized and degraded were then measured according to the method of Bihain, B.E., and Yen, F.T. (1992). Free fatty acids activate a high-affinity saturable pathway for degradation of low-density lipoproteins in fibroblasts from a subject homozygous for familial hypercholesterolemia. Biochemistry *31*, 4628-4636.

Results:

The PLC cell line displayed a much greater capacity to bind, internalize and degrade ^{125}I-LDL in the presence of increasing concentrations of oleate, as compared to the HepG2 cell line (Fig. 24). This is most marked in the degradation. The decrease in degradation observed with > 0.5 mM oleate concentrations is thought to be due to the accumulation of oleate as triglycerides in the cells. This increase in lipid in the cells decreases proteolytic degradation at the lysosomal level.

Quantitative PCR and facs data indicates that LSR expression is almost 50% higher in HepG2 cells than in PLC cells. This would be consistent with the notion of compensation for the lower activity of the receptor in the cells.

These *in vitro* data suggest that a person with a G/G genotype (hence Ser) would display a greater ability to clear triglycerides during the postprandial stage as compared to one with a G/A genotype. Since we have postulated a rate-limiting role of LSR in the removal of dietary lipid, these data could explain the significant association found between low postprandial triglyceride levels and G/G genotype. In contrast to G/G homozygotes, G/A heterozygotes with lower LSR activity would have a lesser capacity of removing dietary lipid, thus increasing their time in the circulation. This would in turn cause a change in the partitioning of lipid between the liver and the adipose tissue, leading to a greater deposition of fat in the adipose tissue.

This example indicates the potential use of this polymorphism, as a marker to detect people with a propensity towards obesity. It also supports the hypothesis that LSR is a potential pharmaceutical target for the development of compounds aimed at targeting lipids away from the adipose tissue and towards the liver.

Example 18: Leptin Transport through the Blood Brain Barrier

Human leptin transport through the blood-brain barrier (BBB) is studied using an in vitro model (Dehouck, et al J Neurochem 54:1798-801, 1990 hereby incorporated herein by reference in its entirety including any figures, tables, or drawings). This model closely mimics the *in vivo* situation with regard to the selective passage of nutrients and drugs through the cerebro-vascular endothelium. The presence of tight junctions that prevent non-specific diffusion, the expression of specific receptors such as LDL receptor and transferrin receptor, and the expression of P-glycoprotein in brain capillary endothelial cells *in vitro* demonstrates that this model is a useful system to study the selective transport through the BBB. Briefly, this model consists of a co-culture of bovine brain capillary endothelial cells (ECs) and rat astrocytes (Figure 31). The astrocytes are seeded on the plastic of a six-well dish and grown for 3 weeks. A collagen-coated filter is then set in each dish and bovine ECs are plated on the upper-side of the filter. ECs form a confluent monolayer in 5 days and they are used for experiments after 16 days of coculture with astrocytes.

Methods

Leptin transcytosis: Experiments were performed on brain capillary endothelial cells in coculture with astrocytes for 16 days. On the day of the experiment, ECs were transferred to a clean 6-well plate containing 2 mL of Ringer-Hepes buffer (*see*, Fig. 32). At time 0, 1 mL Ringer Hepes containing ^{125}I-leptin was placed in the upper compartment. After 30, 60, 120, or 180 min incubation at 37 °C on a rocking platform, the insert was transferred into another well of a six-well plate to minimize the possible passage of substances from the lower to the upper compartment. At the end of the experiment, the amount of radioactivity of each well was counted. The transcytosis was performed over 3 h with 1) 10 ng/mL ^{125}I-leptin (10,000 dpm/ng), 2) 10ng/mL ^{125}I-leptin + 1 µg/mL of cold leptin, 3) 10 ng/mL ^{125}I-

leptin + 50 μg/mL peptides or 4) 10 ng/mL ^{125}I-leptin + 2 mg/mL lactoferrin. The synthetic peptides studied include the human (HP) and mouse (MP) leptin peptide fragments : CHLPWASGLETLDSLGGVLEAS (SEQ ID NO:57) and CSLPQTSGLQKPESLDGVLEAS (SEQ ID NO:67), respectively.

Sucrose and inulin permeability studies: The [^{14}C]-sucrose (342Da) and [^{3}H]-inulin (57000 Da) are hydrosoluble molecules which pass through the BBB through non-receptor mediated processes. The transport is nonspecific and primarily through tight junctions. These serve as markers for the integrity of the BBB and hence toxicity of the added compounds on the cerebral endothelium.

After 16 days of coculture, permeability studies were performed as described in Figure 32. On the day of the experiment, ECs were transferred to a new 6-well plate containing 2 mL of Ringer-Hepes. At time 0, 1 mL Ringer Hepes containing [^{14}C]-sucrose, [^{3}H]-inulin and cold leptin were placed in the upper compartment. Sucrose and inulin permeability studies were performed in the presence of 10 ng/mL leptin, 5 μg/mL leptin, 10 μg/mL leptin or without leptin as a control. The effect of peptides was also tested by the addition of 10 ng/mL leptin+50 μg/mL mouse peptide (MP), 10 ng/mL leptin+50 μg/mL human peptide (HP), or 10 ng/mL leptin+2 mg/mL lactoferrin (lacto). At the end of the experiment, an aliquot from each well was placed in a scintillation vial, and radioactivity was determined.

The transport of molecules through the endothelial monolayer was determined for each time point as % passage: % passage of radiolabelled molecule through the endothelium: dpm found in the lower compartment at a time point divided by the initial dpm found in the upper compartment: % transport at 30min = (lower dpm t30 / upper dpm)*100

Results

Figure 33 shows an increased transport of radiolabelled leptin over time through the endothelium monolayer after 16 days of coculture. The addition of unlabelled leptin reduced the amount of leptin by approximately 30%, indicating that there is a specific component involved in the transport of leptin across the EC monolayer. A higher concentration of unlabelled leptin is needed to decrease the effect of nonspecific processes. The specific component involved in leptin transport is associated with the complete differentiation and formation of the BBB.

Lactoferrin, an inhibitor of LSR, significantly inhibited the amount of leptin transported. The mouse leptin peptide fragment had no significant effect on leptin transport. However, the addition of human leptin peptide fragment caused a significant increase in the amount of leptin transcytosis. This same peptide fragment increases LSR activity in human hepatocytes.

The integrity of the BBB was tested using sucrose and inulin (Figure 34A and 34B). It is clear that the integrity of the BBB was not significantly compromised by the addition of leptin, the peptides, or

lactoferrin. Hence, we can conclude that the trancytosis measured in Figure 33 represents active processes, and is not due to disintegration of the EC monolayer.

Thus the invention is drawn to inhibitors and activators of LSR as a means for controlling the transport of leptin across the blood brain brain. Agents directed towards activation or inhibition of brain LSR regulate leptin transport into the CNS where it acts as satiety factor.

While preferred embodiments of the invention has been illustrated and described, it will be appreciated that various changes can be made by one skilled in the art without departing from the spirit and scope of the invention.

Example 19: Effect of Longterm Exposure to High Levels of Leptin on LSR Activity

Human liver cells preincubated with 200 ng/mL human recombinant leptin for 24 h had a markedly reduced LSR activity (Fig. 35A, □), as compared to those not preincubated with leptin (Fig. 35A, ■). Leptin retained its ability to acutely increase oleate-induced ^{125}I-LDL binding to LSR in a subsequent short incubation (Fig. 35A, □). However, the maximal stimulatory effect was reduced by about 50%, and was achieved only with higher leptin concentrations (100 ng/mL). In hepatocytes preincubated for 24 h with high doses of leptin (200-400 ng/mL), a 25-35% decrease of hepatocyte LSR mRNA relative to GAPDH was observed, as compared to control cells (Fig. 35B).

Although not wishing to be limited by any particular theory, these data suggest that the consistently elevated leptin levels in *db/db* mice cause a decrease in LSR expression, as well as cause a reduction in leptin's ability to acutely stimulate the receptor. This, and the fact that plasma leptin did not increase in db^{Pas}/db^{Pas} after the test meal could explain the massively-elevated postprandial lipemic response observed in this strain. However, because leptin signaling to LSR proceeds independent of the Ob-R, acute increase in plasma leptin concentrations obtained with injection of 500-50,000 ng of leptin in *db/db* mice could accelerate the removal of lipid by activating LSR.

Based on these observations, it is likely that 1) the reduced LSR activity, caused by the constantly high levels of circulating leptin, and 2) the lack of increase in plasma leptin levels during the postprandial stage, contribute to elevated postprandial plasma TG levels in *db/db*. It should be noted that the dose of leptin regulating postprandial lipemia in *ob/ob* is ≃ 500 fold lower than those typically used to reduce food intake (2). In *db/db* mice, leptin doses 10 fold greater than those used in *ob/ob* mice were needed to achieve maximal regulation of postprandial lipemia. Thus, the regulation of postprandial lipemia in *db/db* mice appears partially leptin-resistant, despite the fact that leptin signaling effect occurs independently of the Ob-R.

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What is claimed is :

CLAIMS

1. A leptin polypeptide fragment that modulates an activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of a polypeptide sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39, wherein said at least 4 and not more than 50 contiguous amino acids include the leptin fragment central sequence.

2. A polynucleotide encoding said leptin fragment of claim 1, or the complement of a polynucleotide encoding said leptin fragment of claim 1.

3. A recombinant vector comprising said polynucleotide of claim 2.

4. A recombinant cell comprising said polynucleotide of claim 2.

5. A pharmaceutical composition comprising said leptin fragment of claim 1 and a pharmaceutically acceptable diluent.

6. A method of preventing or treating an obesity-related disease or disorder comprising providing to an individual in need of such treatment said pharmaceutical composition of claim 5.

7. The method of claim 6, wherein said obesity-related disease or disorder is selected from the group consisting of obesity, anorexia, cachexia, cardiac insufficiency, coronary insufficiency, stroke, hypertension, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, hyperuricemia, congenital lipodystrophy, and Syndrome X.

8. A method of designing mimetics of a leptin fragment that modulates an activity of LSR, comprising:

(a) identifying critical interactions between one or more amino acids of said leptin fragment of claim 1 and LSR;

(b) designing potential mimetics to comprise said critical interactions; and

(c) testing said potential mimetics abilities to modulate said activity as a means for designing said mimetics.

9. A chimeric oligonucleotide, comprising at least 9 contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16, wherein said at least 9 contiguous nucleotides comprise at least one amino acid codon selected from the group consisting of TTA, TTG, TCA, TCG, TAU, TAC, TGT, TGC, TGG, CAA, CAG, AGA, GAA, GAG, and GGA, and wherein a point mutation is present in said codon such that said codon is a stop codon.

10. A method of inhibiting the expression of at least one subunit of LSR, comprising providing to a cell said oligonucleotide of claim 9.

11. A method of treating or preventing an obesity-related disease or disorder comprising providing to an individual in need of such treatment said oligonucleotide of claim 9.

12. A chimeric oligonucleotide, comprising at least 9 contiguous nucleotides of SEQ ID NO:1, wherein said at least 9 contiguous nucleotides comprise a single nucleotide polymorphism selected from the group consisting of A1 to A32.

13. A method of treating or preventing an obesity-related disease or disorder comprising providing to an individual in need of such treatment said oligonucleotide of claim 12.

14. A zinc finger protein, comprising a DNA binding domain that binds specifically to 18 nucleotides of a sequence at least 50% homologous to SEQ ID NO:1, wherein said 18 nucleotides comprise two fragments of 9 contiguous nucleotides, and wherein said fragments are separated by 0, 1, 2, or 3 nucleotides.

15. A polynucleotide encoding said protein of claim 14.

16. A recombinant vector comprising said polynucleotide of claim 15.

17. A method of treating or preventing an obesity-related disease or disorder comprising providing to an individual in need of such treatment said vector of claim 16.

18. A non-human mammal comprising said polynucleotide of claim 15.

19. A method of modulating the expression of at least one subunit of LSR, comprising providing to a cell said polynucleotide of claim 15 or a fragment of an LSR polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.

20. A method of treating or preventing an obesity-related disease or disorder comprising providing to an individual in need of such treatment said polynucleotide of claim 15.

21. A recombinant cell comprising said polynucleotide of claim 15.

22. A method for selecting a compound useful for the treatment or prevention of an obesity-related disease or disorder, comprising:

(a) contacting said recombinant cell of claim 21 with a candidate compound; and

(b) detecting a result selected from the group consisting of a modulation of an activity of the Lipolysis Stimulated Receptor and modulation of expression of the Lipolysis Stimulated Receptor; as a means for

(c) selecting said compound useful for the treatment or prevention of said obesity-related disease or disorder.

23. The method of claim 22, wherein said contacting further comprises a leptin polypeptide fragment that modulates an activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of a polypeptide sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39, wherein said at least 4 and not more than 50 contiguous amino acids include the leptin fragment central sequence.

24. The recombinant cell of claim 21, wherein said recombinant cell is transfected with at least one LSR polypeptide comprising a sequence at least 75% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19.

25. A method for selecting a compound useful for the treatment or prevention of an obesity-related disease or disorder, comprising:

(a) contacting said recombinant cell of claim 24 with a candidate compound; and

(b) detecting a result selected from the group consisting of a modulation of an activity of the Lipolysis Stimulated Receptor and modulation of expression of the Lipolysis Stimulated Receptor; as a means for

(c) selecting said compound useful for the treatment or prevention of said obesity-related disease or disorder.

26. The method of claim 25, wherein said contacting further comprises a leptin polypeptide fragment that modulates an activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of a polypeptide sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39, wherein said at least 4 and not more than 50 contiguous amino acids include the leptin fragment central sequence.

27. A method of selecting for genes that modulate an activity of the Lipolysis Stimulated Receptor, comprising :

(a) providing a retroviral gene library to cells that express said Lipolysis Stimulated Receptor;

(b) contacting said cells with a ligand of said Lipolysis Stimulated Receptor ;

(c) detecting a change in said activity of the Lipolysis Stimulated Receptor as a means for selecting for said genes.

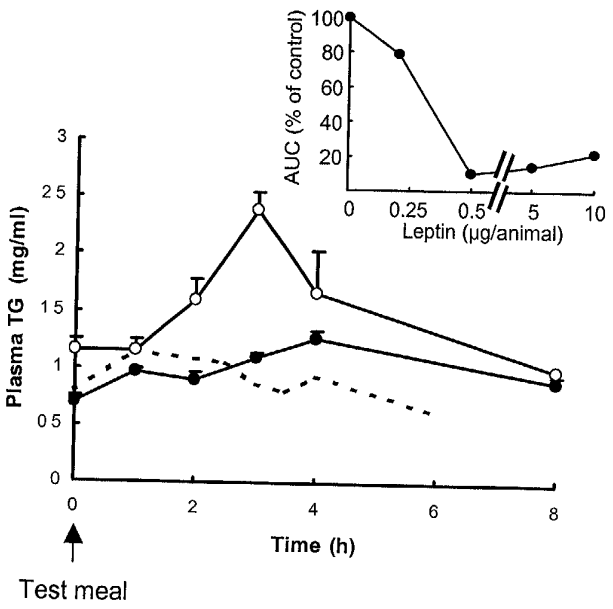
28. The method of claim 27, wherein said ligand is a leptin polypeptide fragment that modulates an activity of LSR, comprising at least 4, but not more than 50 contiguous amino acids of a polypeptide sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39, wherein said at least 4 and not more than 50 contiguous amino acids include the leptin fragment central sequence.

ABSTRACT

The present invention is drawn to methods of screening for new compounds for the treatment of obesity and obesity-related diseases and disorders, as well as methods of treating obesity-related diseases and disorders, based on the discovery of the role of the leptin-LSR interaction in obesity.

5

1A *db/db*



1B *db^{Pas}/db^{Pas}*

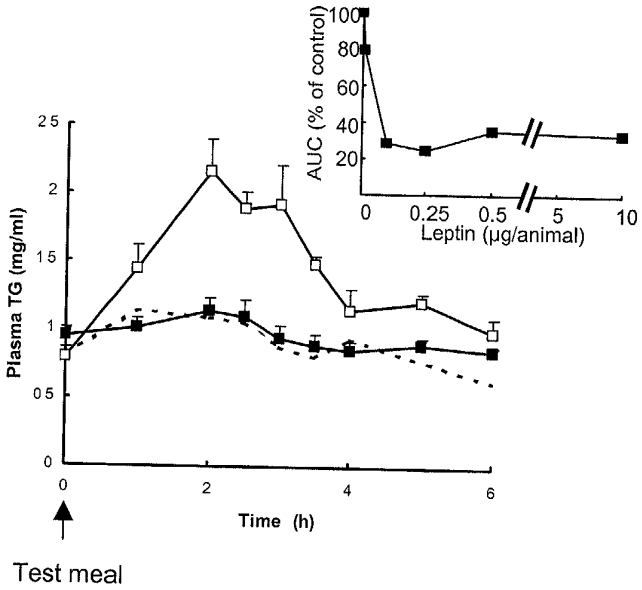


Figure 1

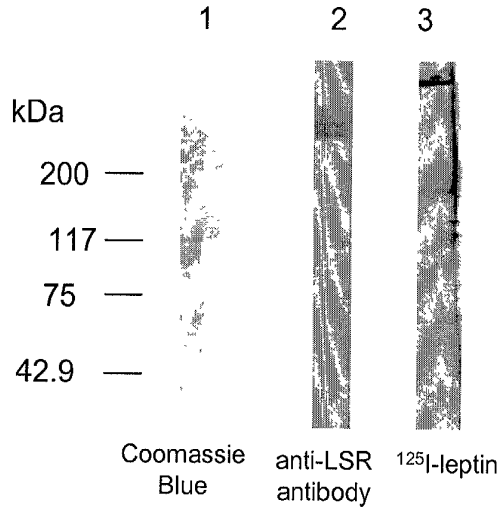
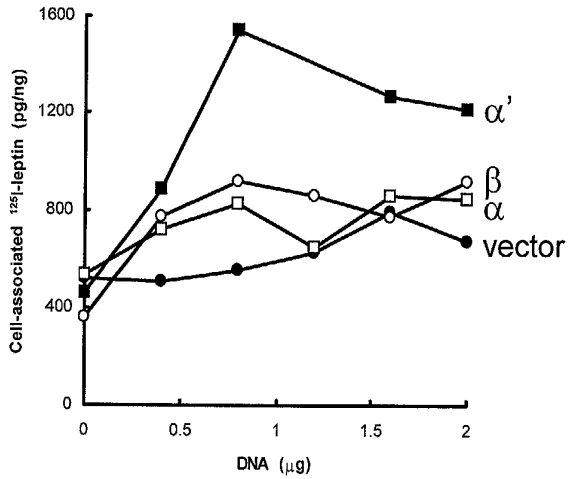
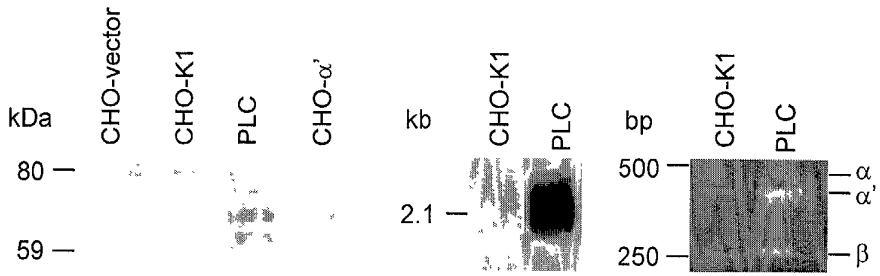

1
2
3
kDa
200
117
75
42.9
Coomassie Blue
anti-LSR antibody
125I-leptin

Figure 2

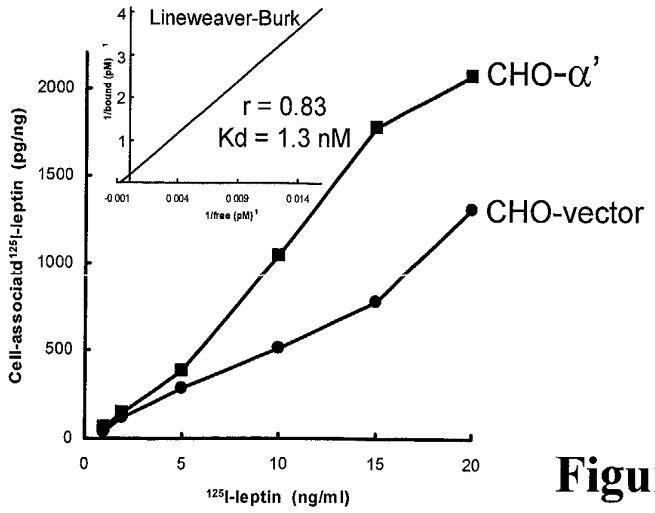
A. Transient transfection



B.



C. α' stable transfection: ^{125}I-leptin binding



D. α' stable transfection: ^{125}I-leptin degradation

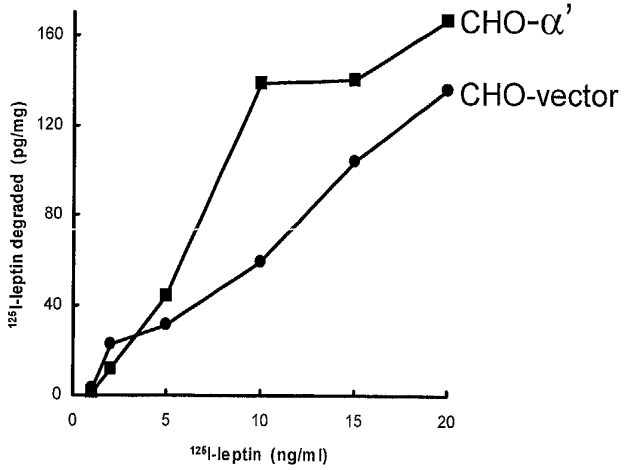
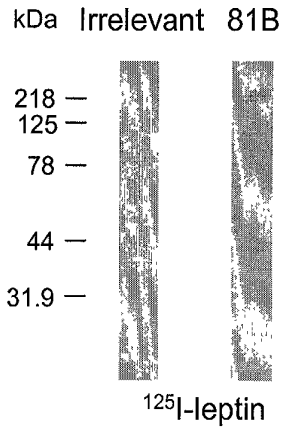
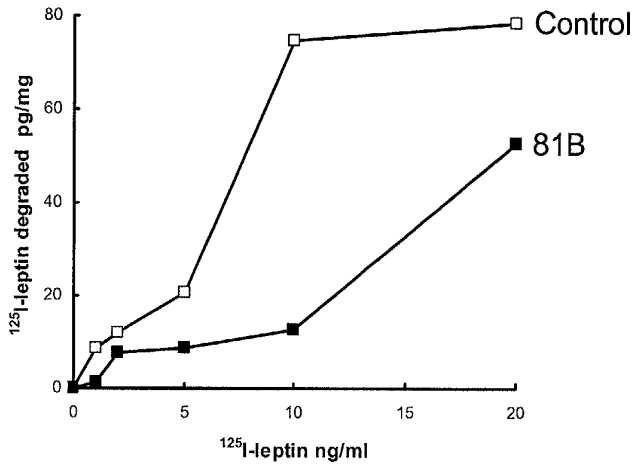


Figure 3

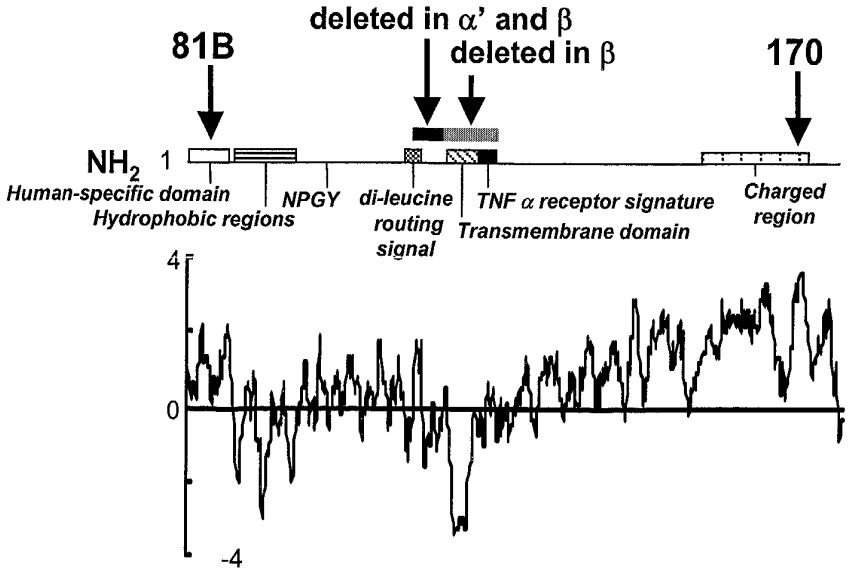
A. ^{125}I-leptin binding



B. ^{125}I-leptin degradation



C. Schematic diagram of human LSR protein motifs



D. FACS Analysis

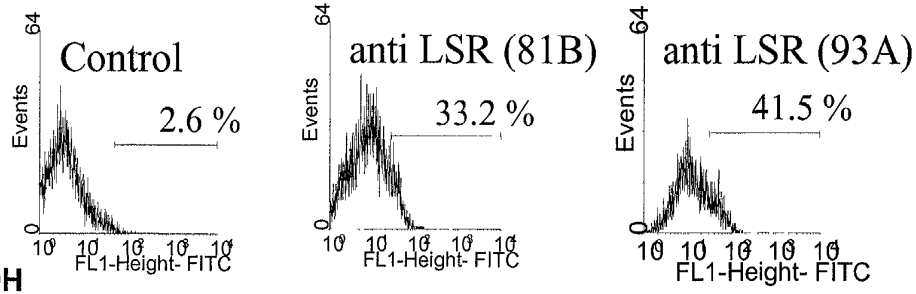


Figure 4

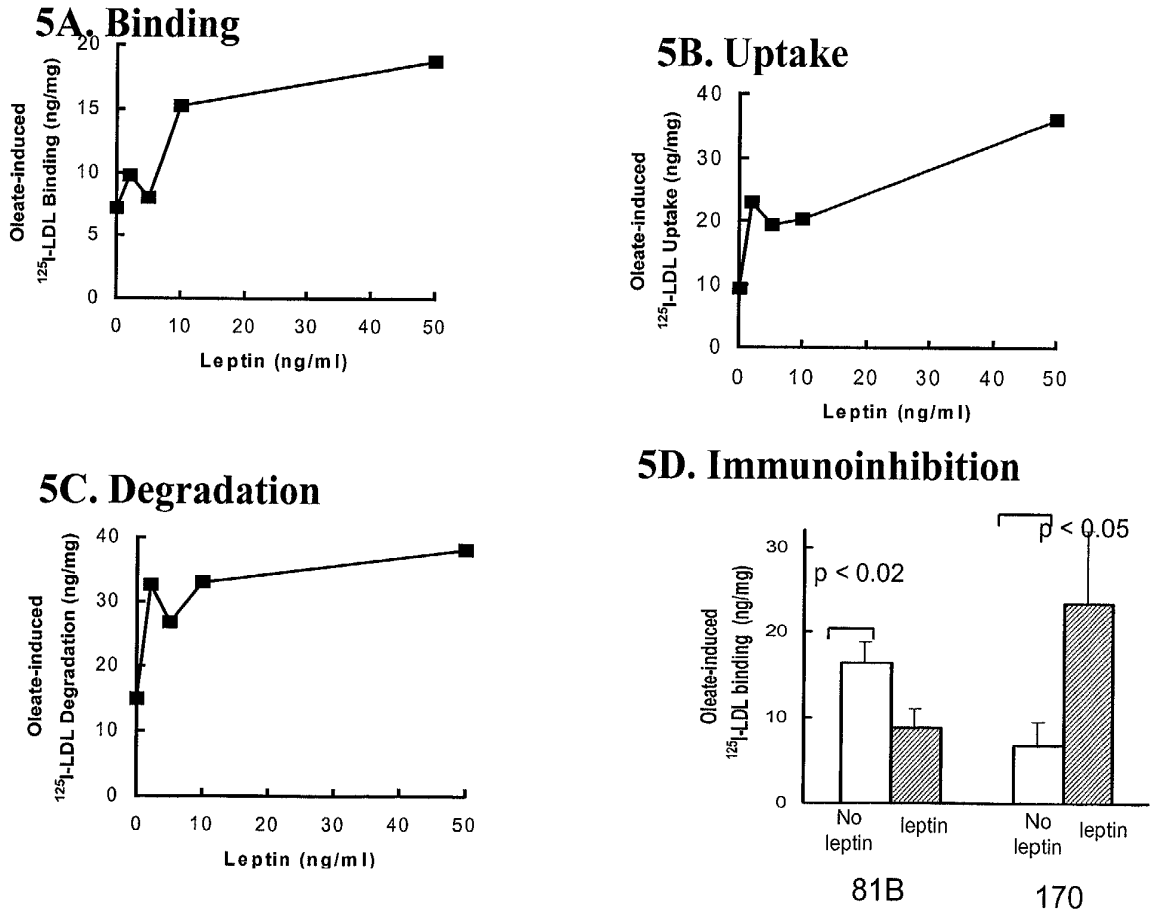
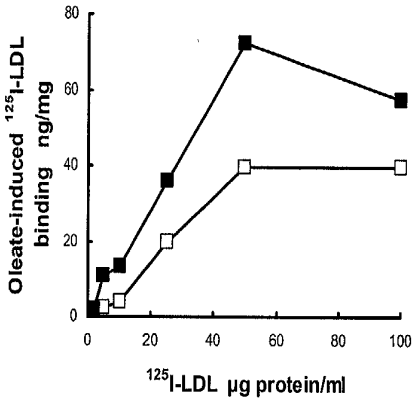
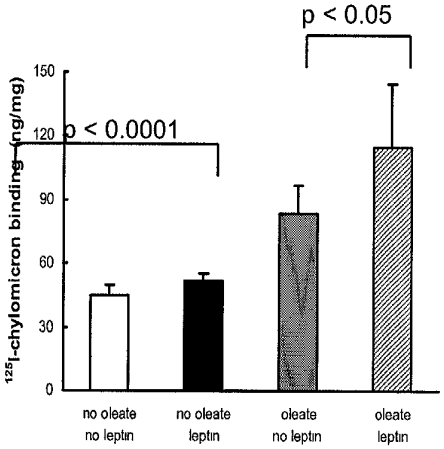

5A. Binding
Oleate-induced
125I-LDL Binding (ng/mg)
Leptin (ng/ml)
5B. Uptake
Oleate-induced
125I-LDL Uptake (ng/mg)
Leptin (ng/ml)
5C. Degradation
Oleate-induced
125I-LDL Degradation (ng/mg)
Leptin (ng/ml)
5D. Immunoinhibition
Oleate-induced
125I-LDL binding (ng/mg)
p < 0.02
p < 0.05
No leptin
leptin
No leptin
leptin
81B
170

Figure 5

6.A
LDL



6.B
Chylomicrons



6.C
Antibody

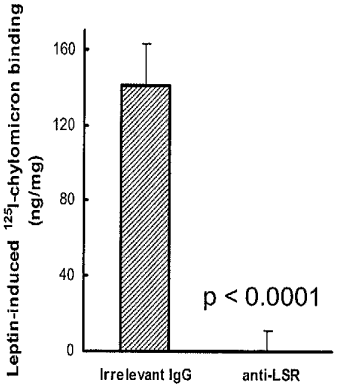
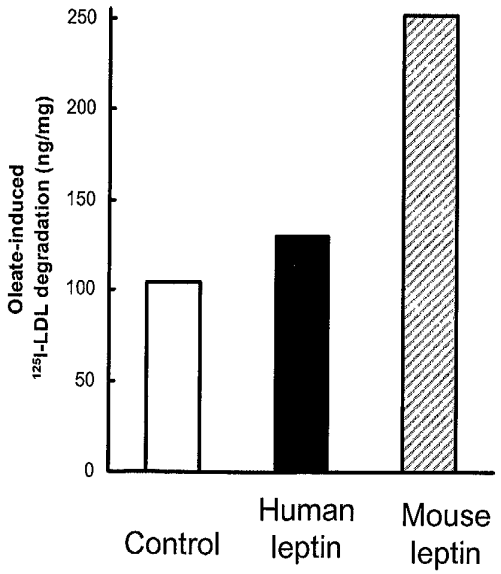


Figure 6

7A. Rat hepatocytes



7B. db^{Pas}/db^{Pas} postprandial plasma TG

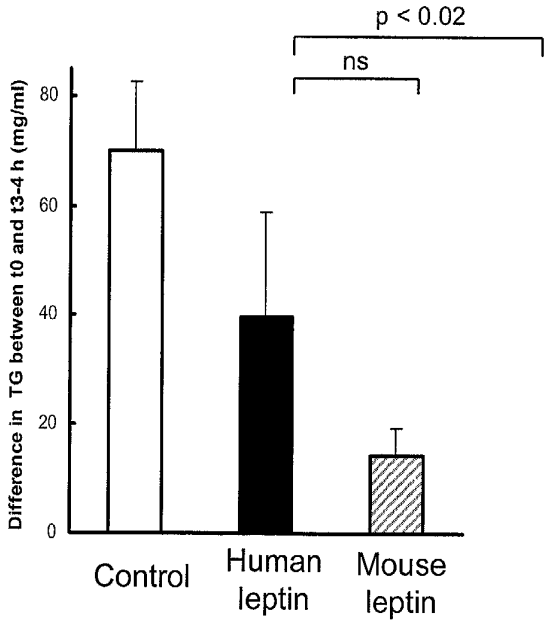


Figure 7

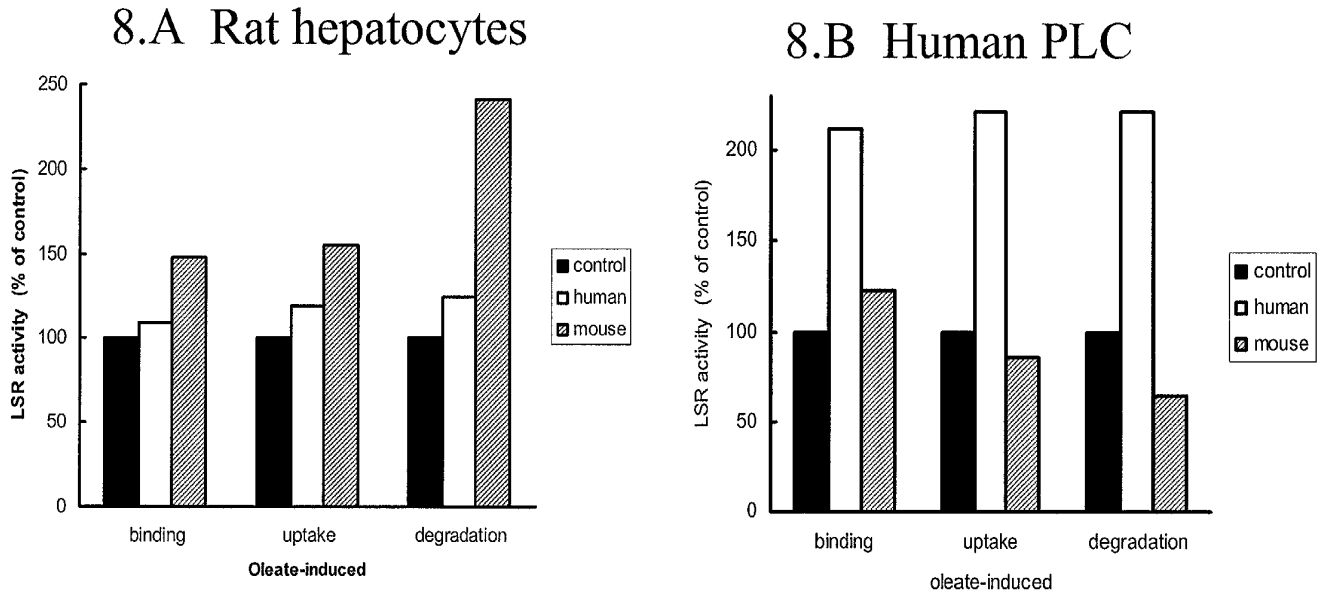
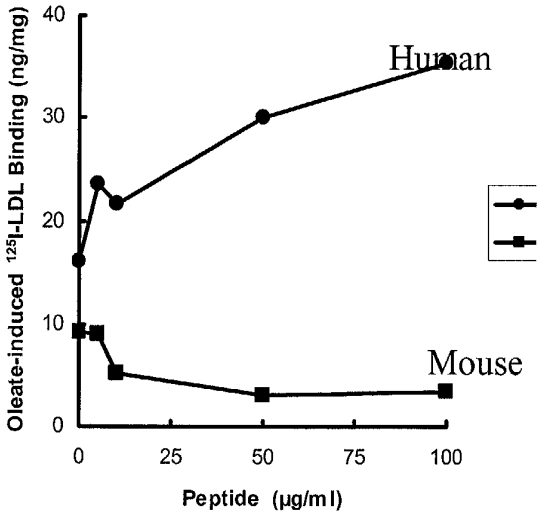

8.A Rat hepatocytes
LSR activity (% of control)
250
200
150
100
50
0
binding
uptake
degradation
Oleate-induced
control
human
mouse
8.B Human PLC
LSR activity (% of control)
200
150
100
50
0
binding
uptake
degradation
oleate-induced
control
human
mouse

Figure 8

9.A Binding



9.B Degradation

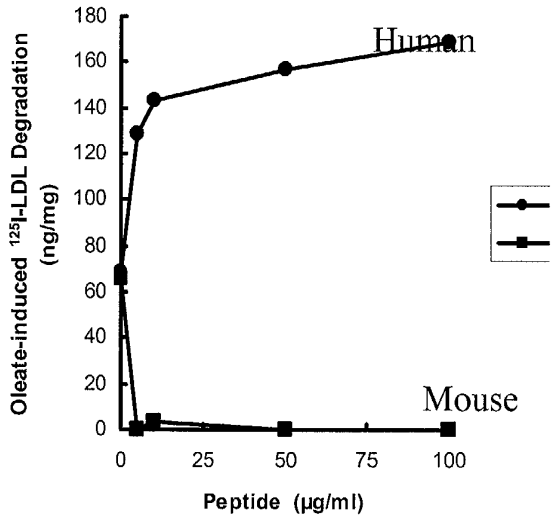
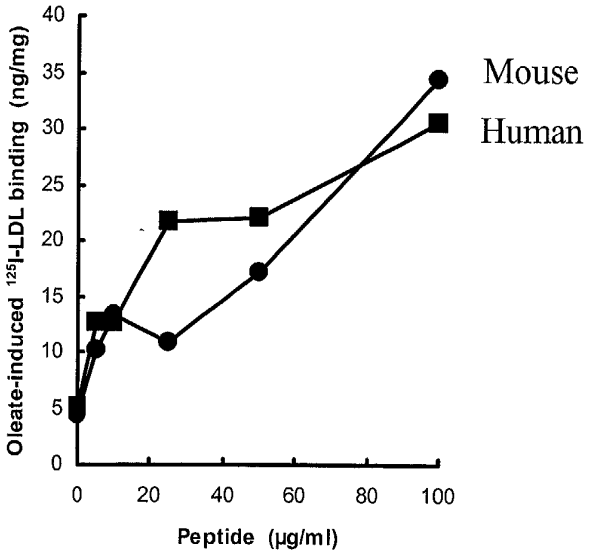


Figure 9

10.A Binding



10.B Degradation

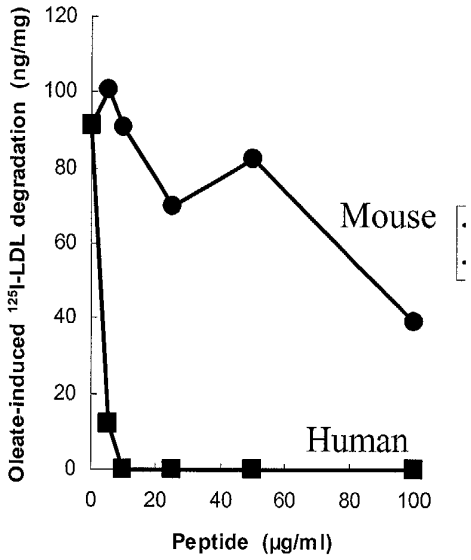


Figure 10

Effect of mouse leptin (A) or leptin peptide (B) on postprandial plasma TG response in ob/ob mice.

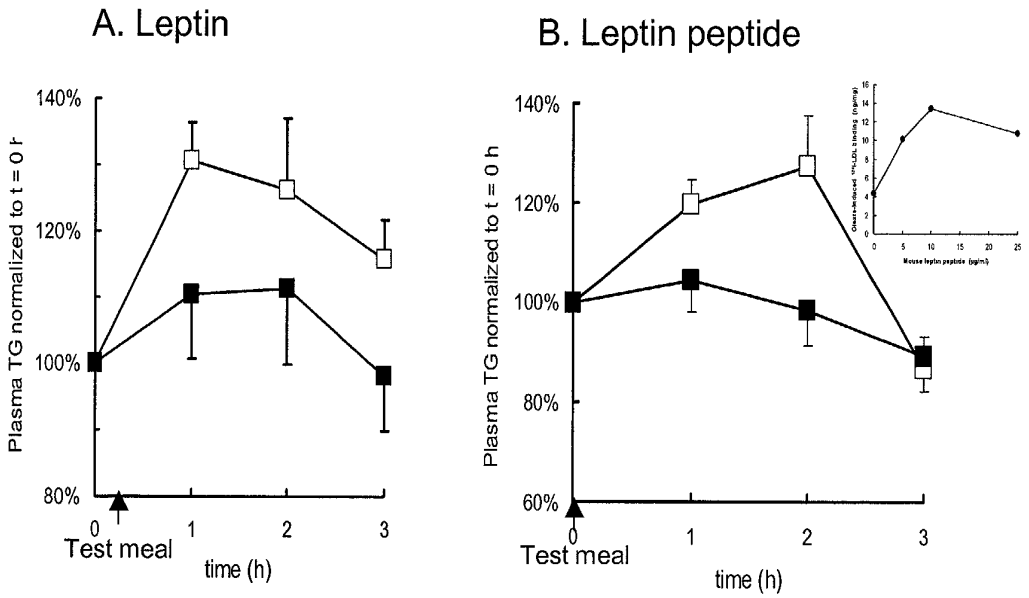


Figure 11

Effect of test meal with and without leptin injection on postheparin lipolytic activity in db^{Pas}/db^{Pas} mice

	Postheparin lipolytic activities in db^{Pas}/db^{Pas} (µmol FFA/ml/h)
No high-fat test meal	11.7 ± 2.4
High-fat test meal	19.5 ± 9.2 [ns]
High-fat test meal + 50 µg leptin	12.2 ± 2.7 [ns]

ns = not significant).

Figure 12

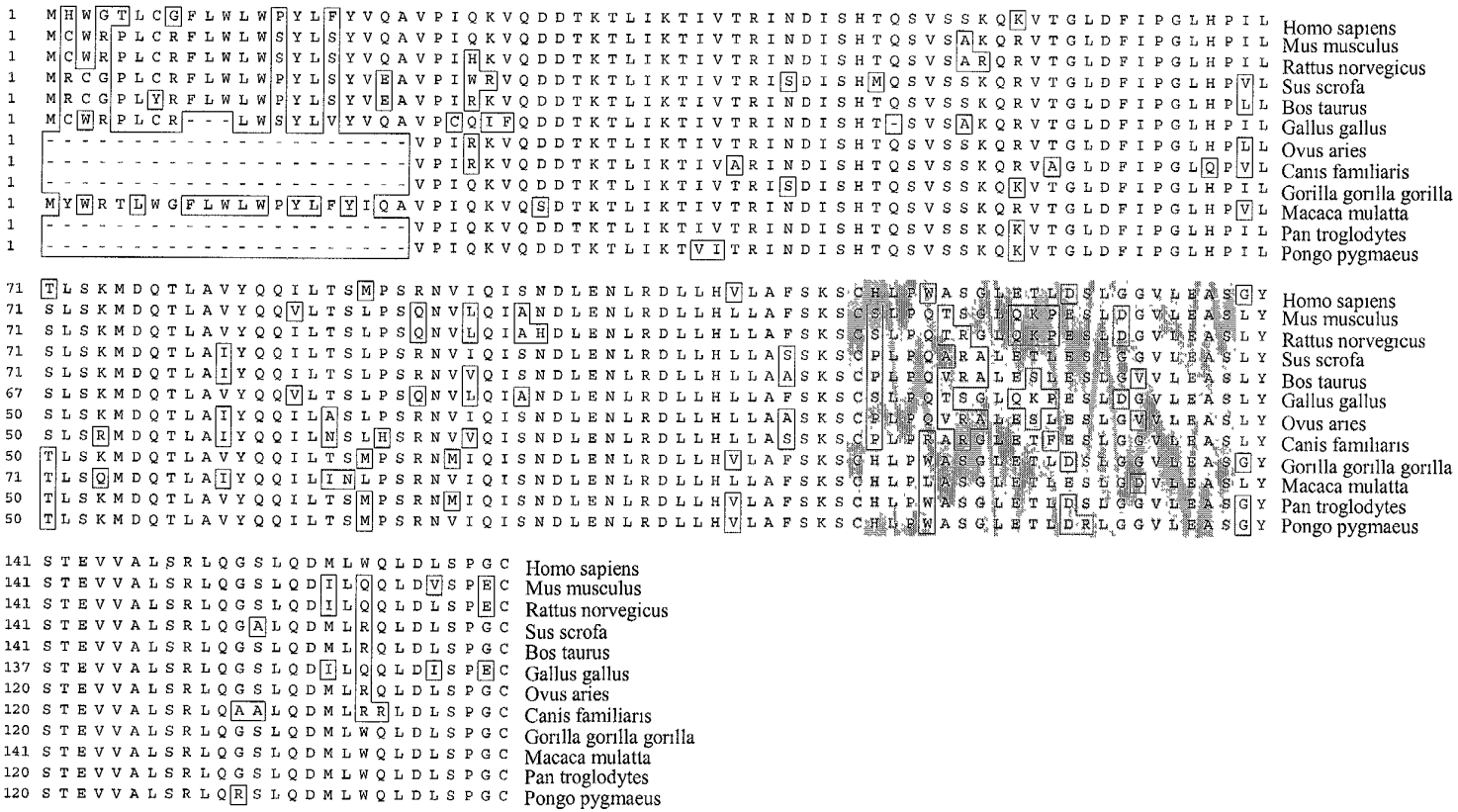

Homo sapiens
Mus musculus
Rattus norvegicus
Sus scrofa
Bos taurus
Gallus gallus
Ovus aries
Canis familiaris
Gorilla gorilla gorilla
Macaca mulatta
Pan troglodytes
Pongo pygmaeus

Figure 13

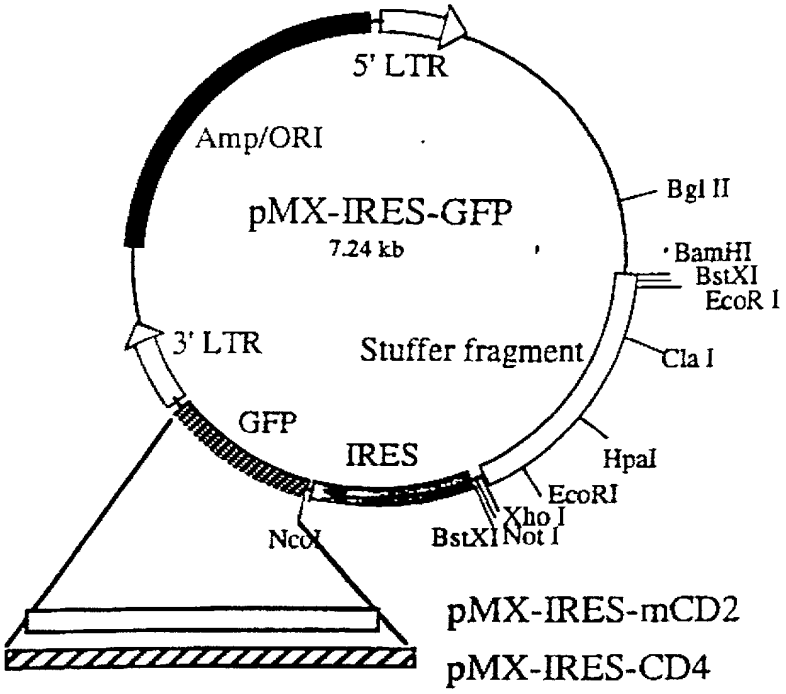
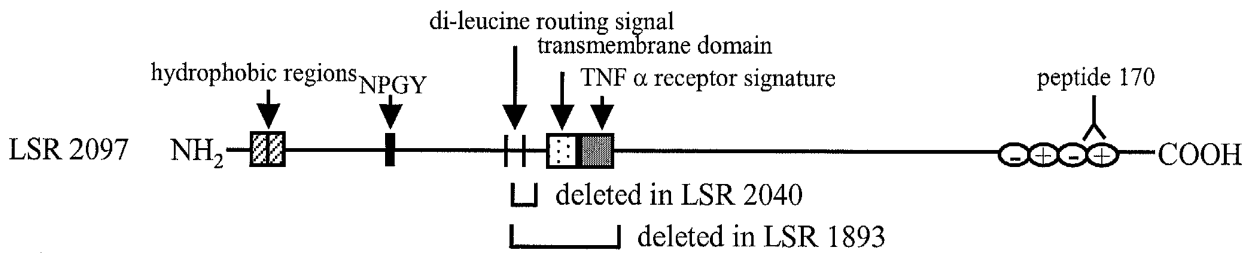

5' LTR
Amp/ORI
pMX-IRES-GFP
7.24 kb
Bgl II
BamHI
BstXI
EcoR I
3' LTR
Stuffer fragment
Cla I
GFP
IRES
HpaI
EcoRI
Xho I
Not I
BstXI
NcoI
pMX-IRES-mCD2
pMX-IRES-CD4

Figure 14

Plan for creation of truncated forms of LSR



1 EcoRI ▭ Sal I
Truncates at AA 145 just prior to the NPGY (nt 437 of the cDNA)

2 EcoRI ▭ SacI
Truncates at AA 234 just prior to exon 4 (nt 703 of the cDNA)

3 Sac I ▭ Xba I
Use alpha so Sac I cuts in exon 4, this effectively deletes the di-leucine signal.
Start at nt 744 which will start at AA 249 and proceed through the C-term.

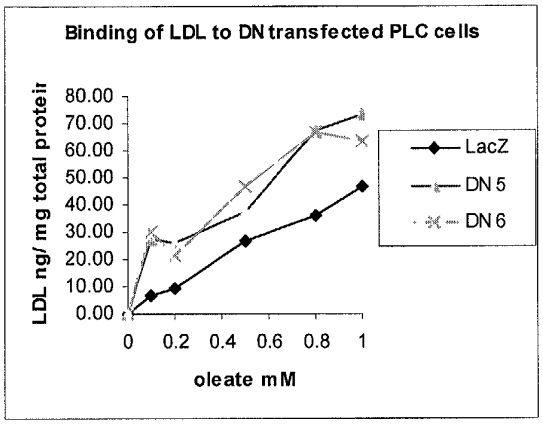
4a-c EcoRI ▭ Kpn I
Truncates at AA 350 (nt 1052) near the end of exon 6
a is from LSR α, **b** is from LSR α' and **c** is from LSR β.

5 Kpn I ▭ Xba I
Contains C-term. end only exon 7- end
AA 353 (nt 1057) -AA 650 (nt 1950 of coding)

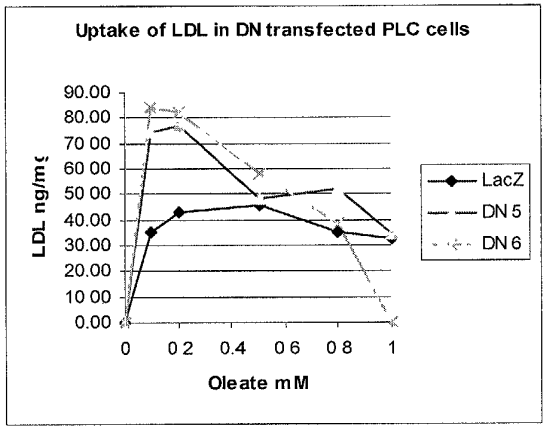
6 Kpn I ▭ Xho I
AA 353 (nt 1057) in exon7 -AA541 (nt 1625) -RSRS domain in exon 9

Figure 15

16A



16B



16C

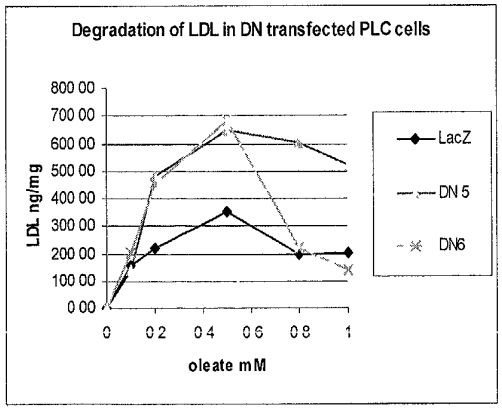
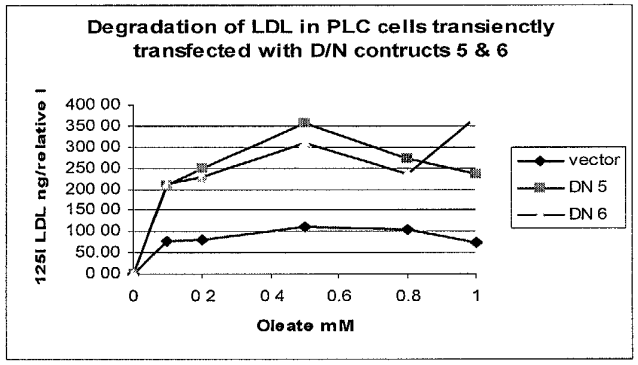
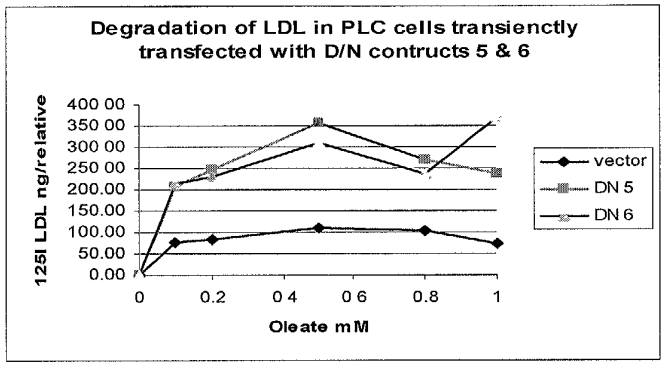


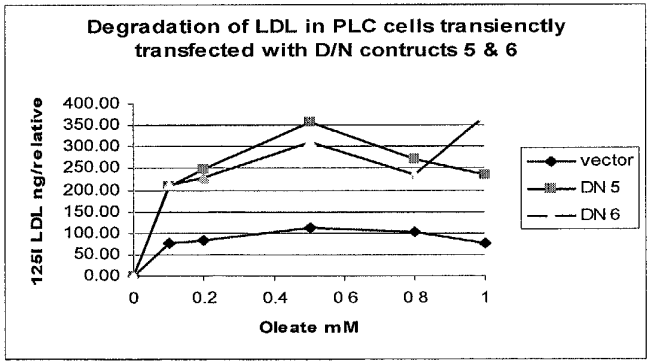
Figure 16



17A



17B



17C

Figure 17

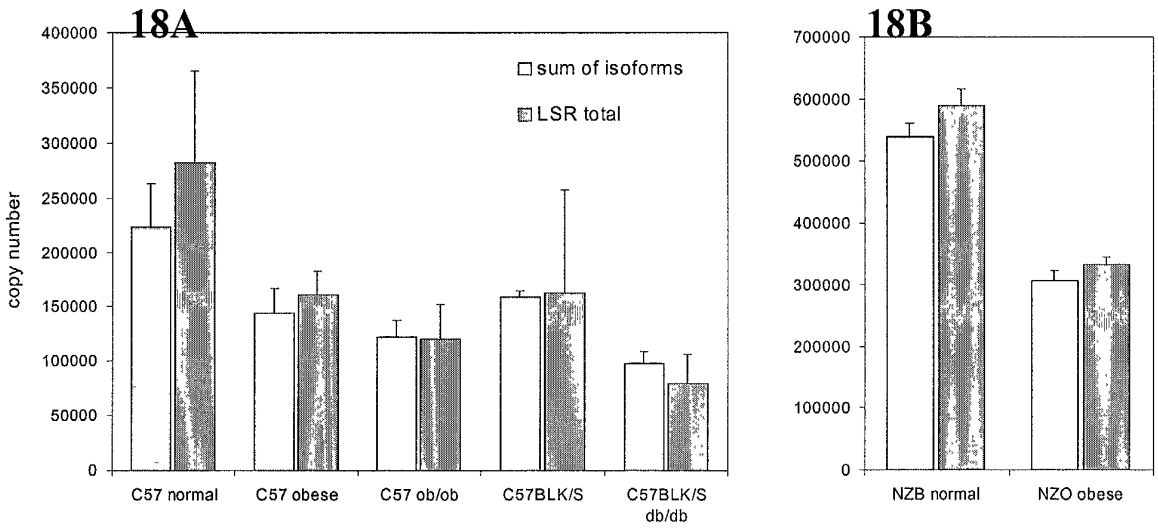

18A
copy number
400000
350000
300000
250000
200000
150000
100000
50000
0
sum of isoforms
LSR total
C57 normal
C57 obese
C57 ob/ob
C57BLK/S
C57BLK/S db/db
18B
700000
600000
500000
400000
300000
200000
100000
0
NZB normal
NZO obese

Figure 18

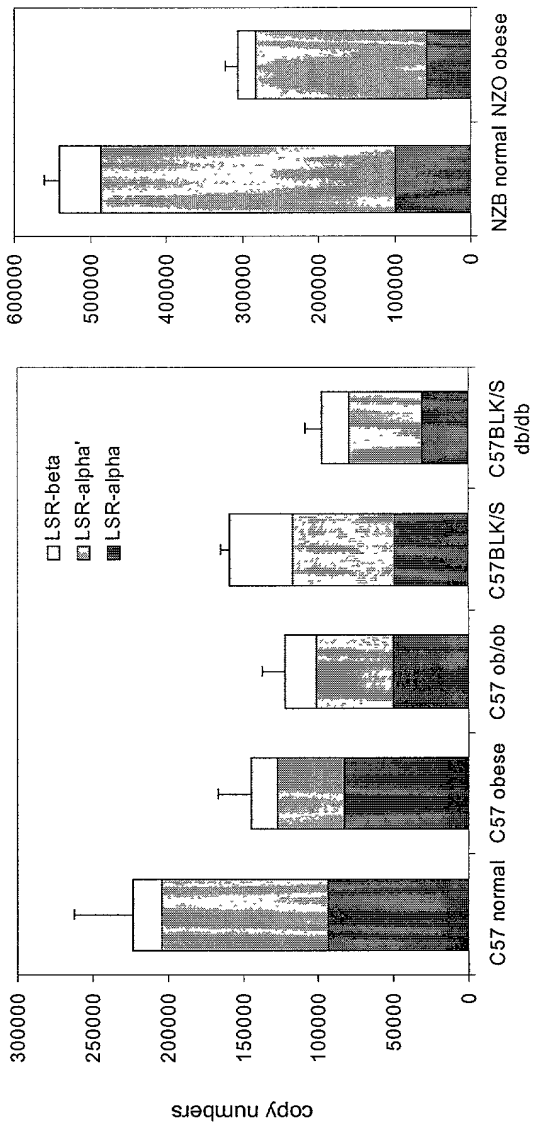
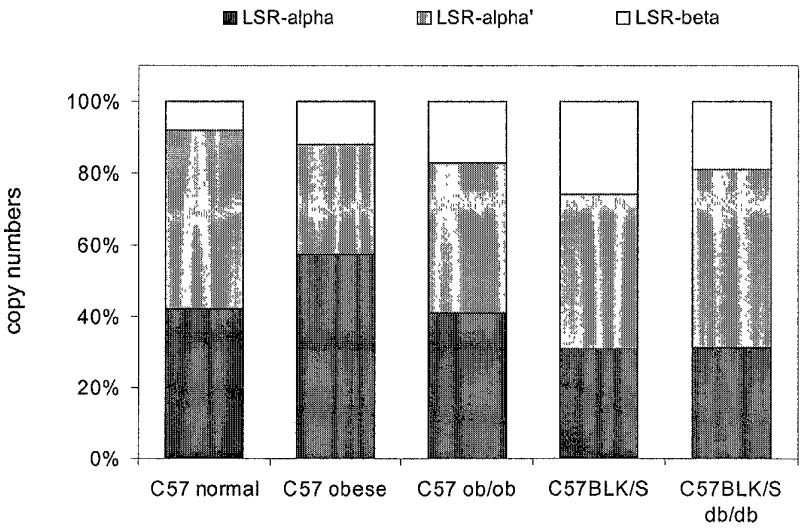

copy numbers
300000
250000
200000
150000
100000
50000
0
LSR-beta
LSR-alpha'
LSR-alpha
C57 normal
C57 obese
C57 ob/ob
C57BLK/S
C57BLK/S db/db
600000
500000
400000
300000
200000
100000
0
NZB normal
NZO obese

Figure 19

20A.



20 B.

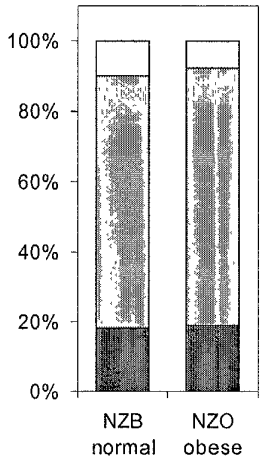


Figure 20

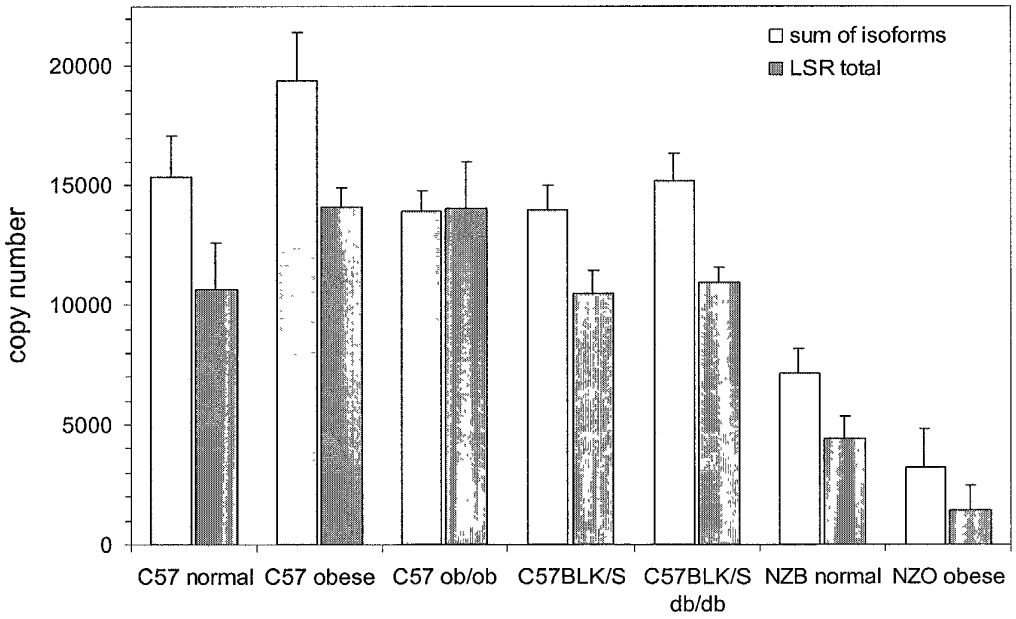

sum of isoforms
LSR total
copy number
20000
15000
10000
5000
0
C57 normal
C57 obese
C57 ob/ob
C57BLK/S
C57BLK/S db/db
NZB normal
NZO obese

Figure 21

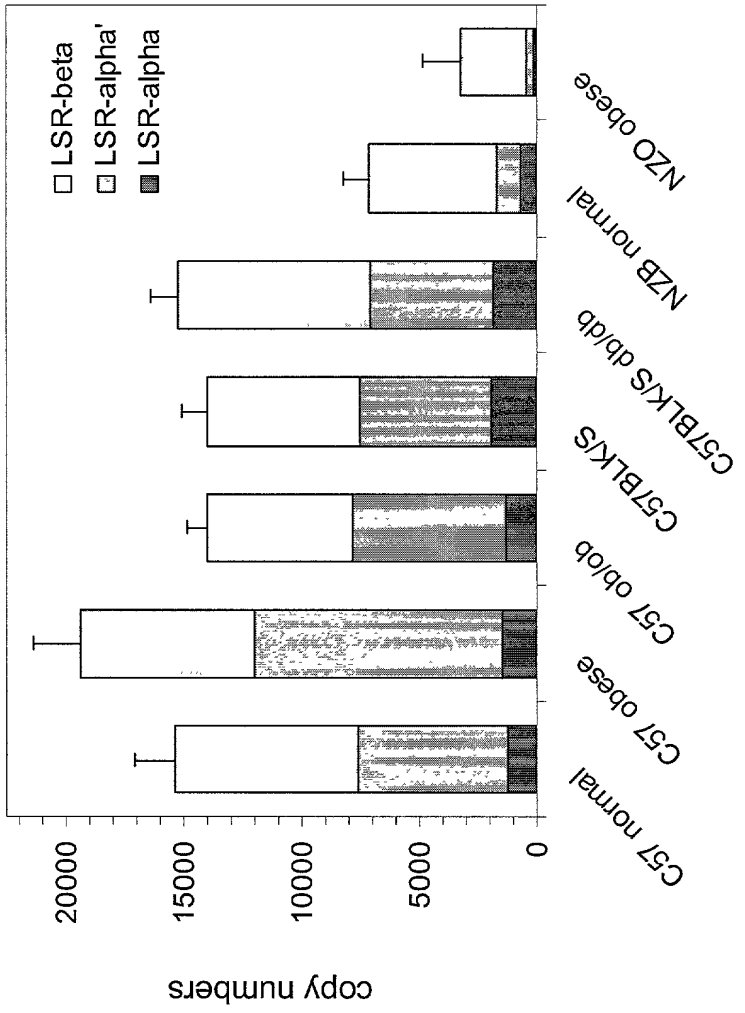

LSR-beta
LSR-alpha'
LSR-alpha
copy numbers
20000
15000
10000
5000
0
C57 normal
C57 obese
C57 ob/ob
C57BLK/S
C57BLK/S db/db
NZB normal
NZO obese

Figure 22

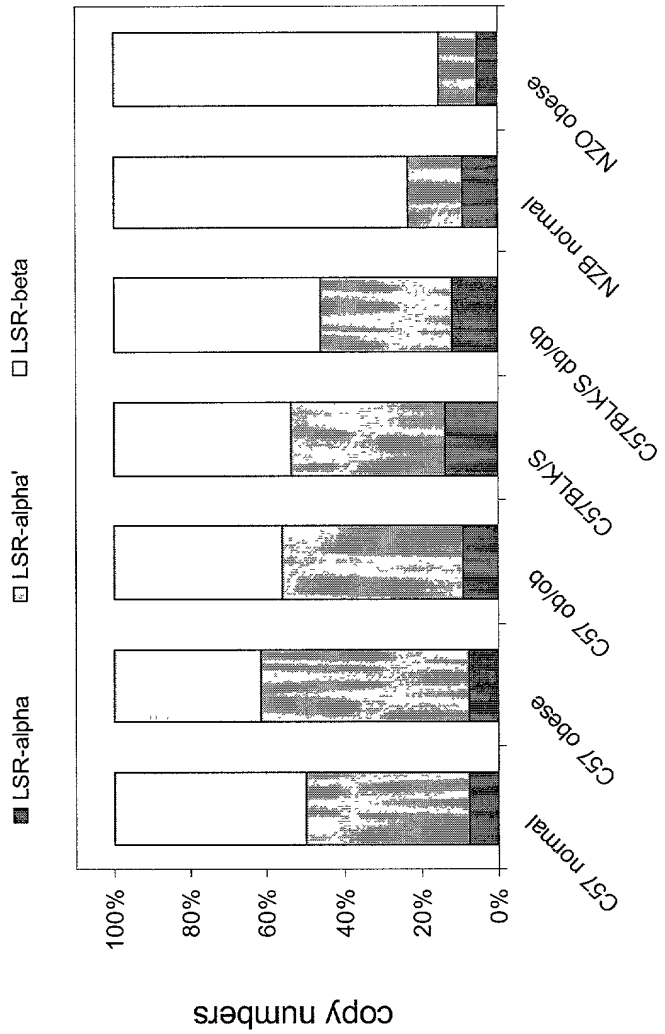

LSR-alpha
LSR-alpha'
LSR-beta
copy numbers
100%
80%
60%
40%
20%
0%
C57 normal
C57 obese
C57 ob/ob
C57BLK/S
C57BLK/S db/db
NZB normal
NZO obese

Figure 23

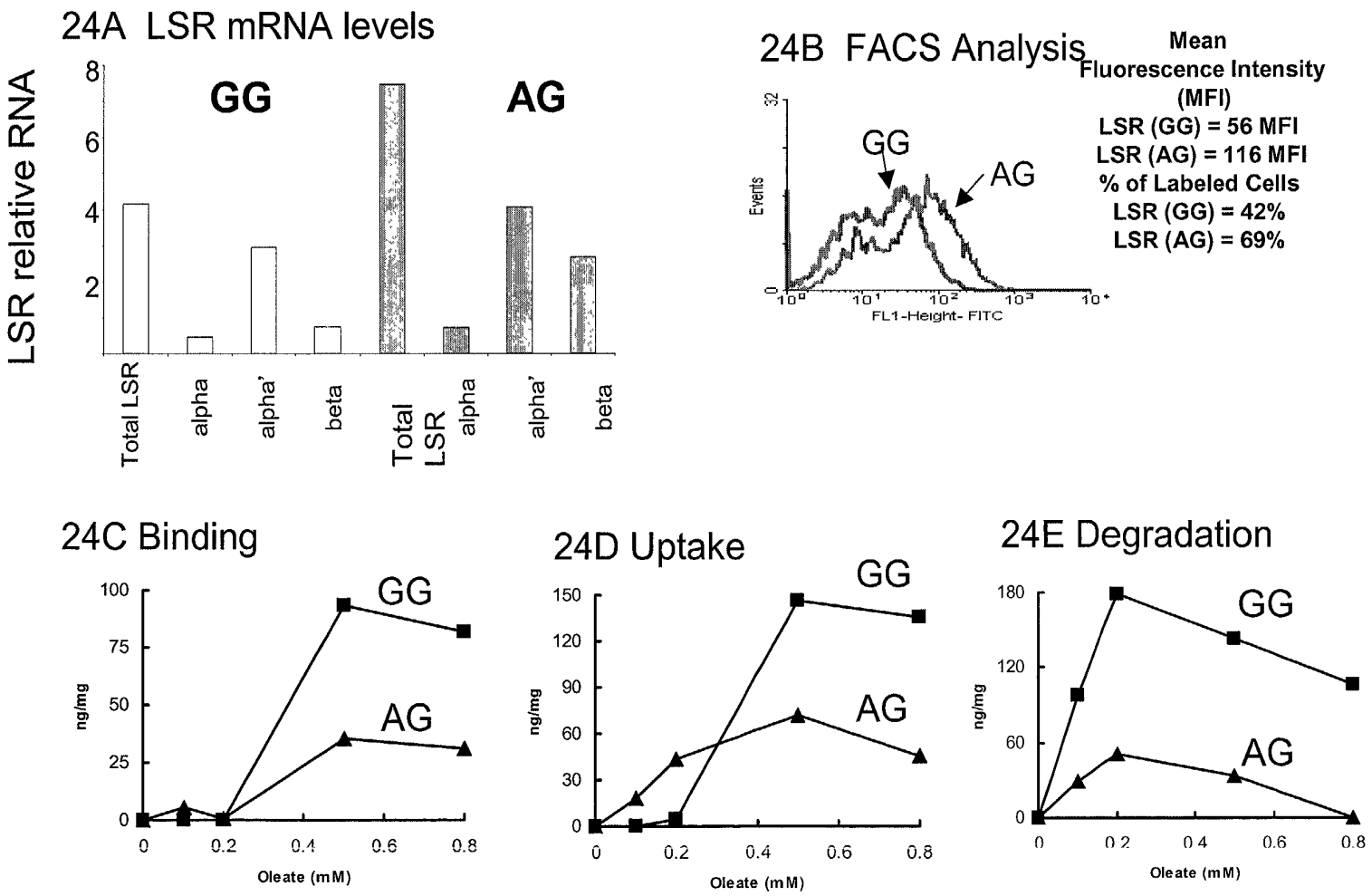

24A LSR mRNA levels
LSR relative RNA
GG
AG
Total LSR
alpha
alpha'
beta
24B FACS Analysis
Mean Fluorescence Intensity (MFI)
LSR (GG) = 56 MFI
LSR (AG) = 116 MFI
% of Labeled Cells
LSR (GG) = 42%
LSR (AG) = 69%
Events
FL1-Height- FITC
24C Binding
24D Uptake
24E Degradation
ng/mg
Oleate (mM)

Figure 24

Table

Characteristics of recombinant ZFPs directed toward LSR sequences.

ID#	ZFP	Fold Activation	Kd (nM)	Target Sequence
5182	2B-1A	21.5	0.10	AAGGTCGCCtatGGTGCAGAC (SEQ ID NO:102)
5183	4A-3A	8.7	0.05	GTGGGAGCCcgGGGGCTGGA (SEQ ID NO:103)
5185	6A-5A	8.4	0.02	TGGGGGTGGGCGGCGGGG (SEQ ID NO:104)
5186	8A-7B	6.5	0.02	CCGGGAGTGcgCAGGGGGTA (SEQ ID NO:105)
5205	1A-7B	29.7	0.30	GTGGCTGCACAAGGTCGCC (SEQ ID NO:106)

Figure 25

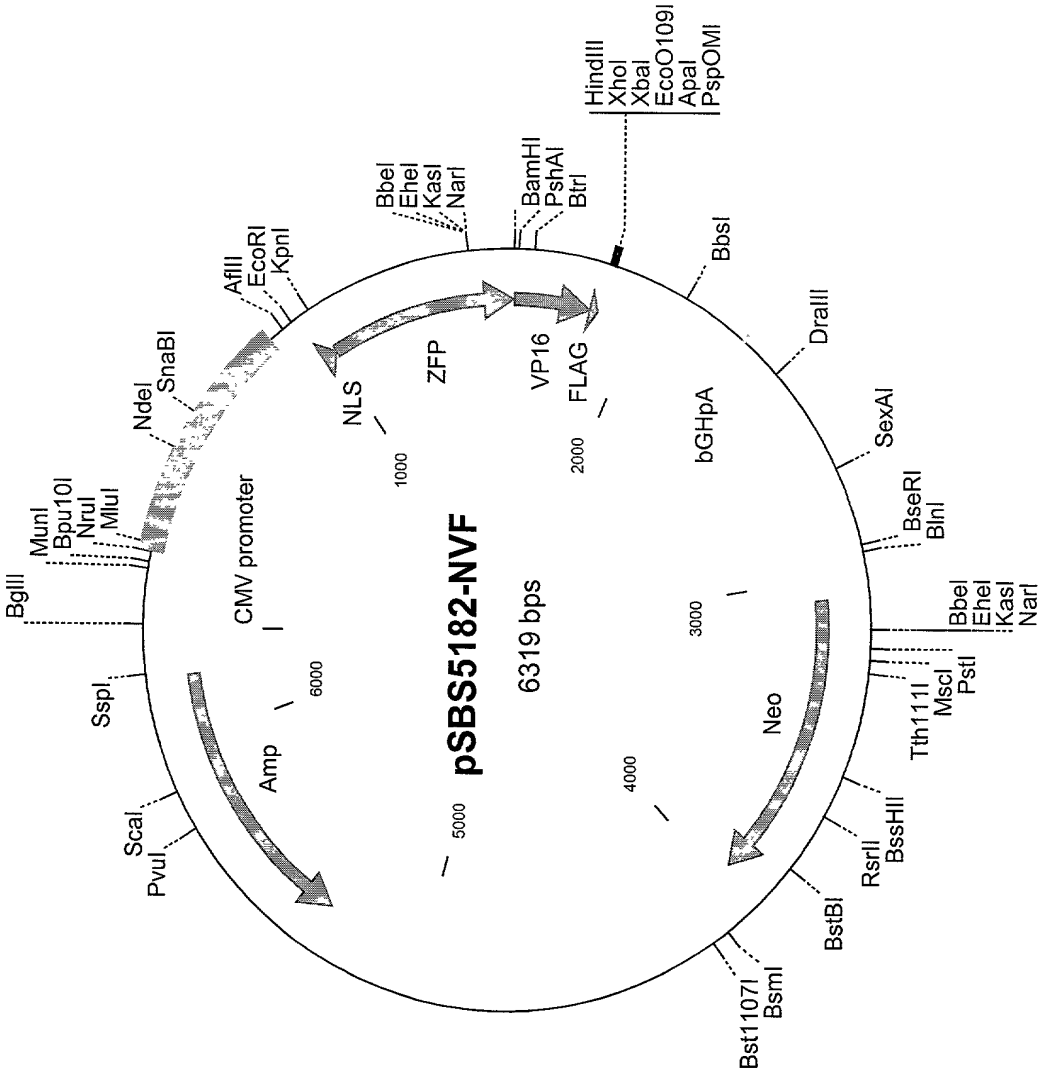

pSBS5182-NVF
6319 bps
CMV promoter
NLS
ZFP
VP16
FLAG
bGHpA
Neo
Amp
1000
2000
3000
4000
5000
6000
BglII
MunI
Bpu10I
NruI
MluI
NdeI
SnaBI
AflII
EcoRI
KpnI
BbeI
EheI
KasI
NarI
BamHI
PshAI
BtrI
HindIII
XhoI
XbaI
EcoO109I
ApaI
PspOMI
BbsI
DraIII
SexAI
BseRI
BlnI
BbeI
EheI
KasI
NarI
MscI
PstI
Tth111I
BssHII
RsrII
BstBI
Bst1107I
BsmI
ScaI
PvuI
SspI

Figure 26A

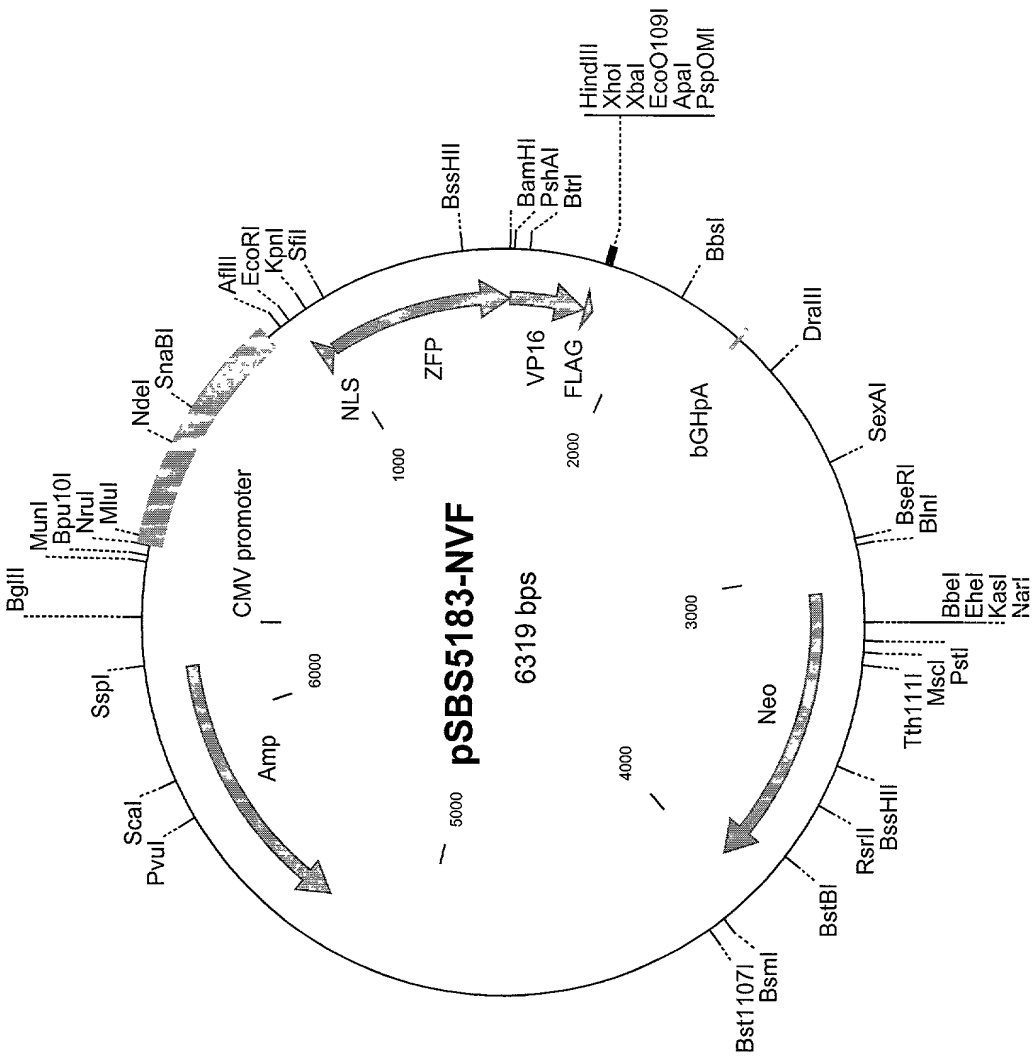

pSBS5183-NVF
6319 bps
CMV promoter
NLS
ZFP
VP16
FLAG
bGHpA
Neo
Amp
1000
2000
3000
4000
5000
6000
BglII
MunI
Bpu10I
NruI
MluI
NdeI
SnaBI
AflII
EcoRI
KpnI
SfiI
BssHII
BamHI
PshAI
BtrI
HindIII
XhoI
XbaI
EcoO109I
ApaI
PspOMI
BbsI
DraIII
SexAI
BseRI
BlnI
BbeI
EheI
KasI
NarI
MscI
PstI
Tth111I
BssHII
RsrII
BstBI
Bst1107I
BsmI
ScaI
PvuI
SspI

Figure 26B

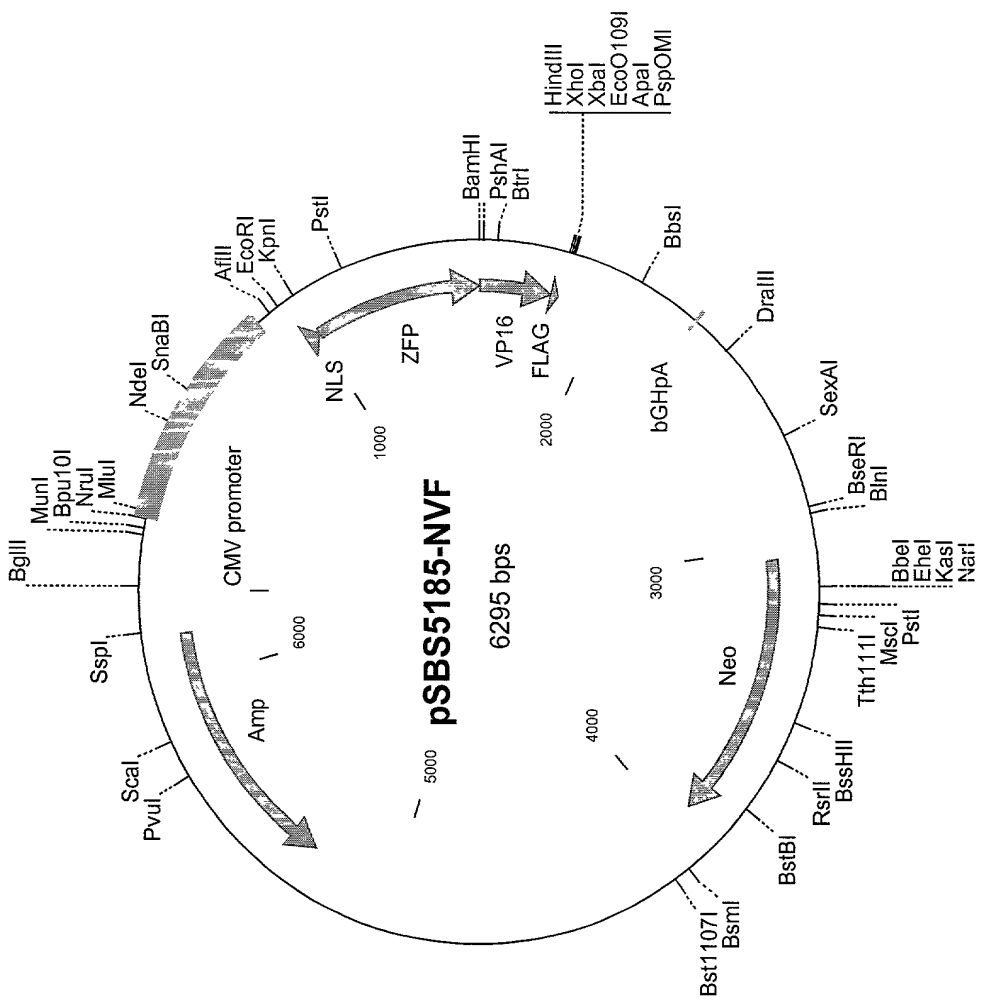

pSBS5185-NVF
6295 bps
CMV promoter
NLS
ZFP
VP16
FLAG
bGHpA
Neo
Amp
1000
2000
3000
4000
5000
6000
BglII
MunI
Bpu10I
NruI
MluI
NdeI
SnaBI
AflII
EcoRI
KpnI
PstI
BamHI
PshAI
BtrI
HindIII
XhoI
XbaI
EcoO109I
ApaI
PspOMI
BbsI
DraIII
SexAI
BseRI
BlnI
BbeI
EheI
KasI
NarI
PstI
MscI
Tth111I
BssHII
RsrII
BstBI
Bsml
Bst1107I
PvuI
ScaI
SspI

Figure 26C

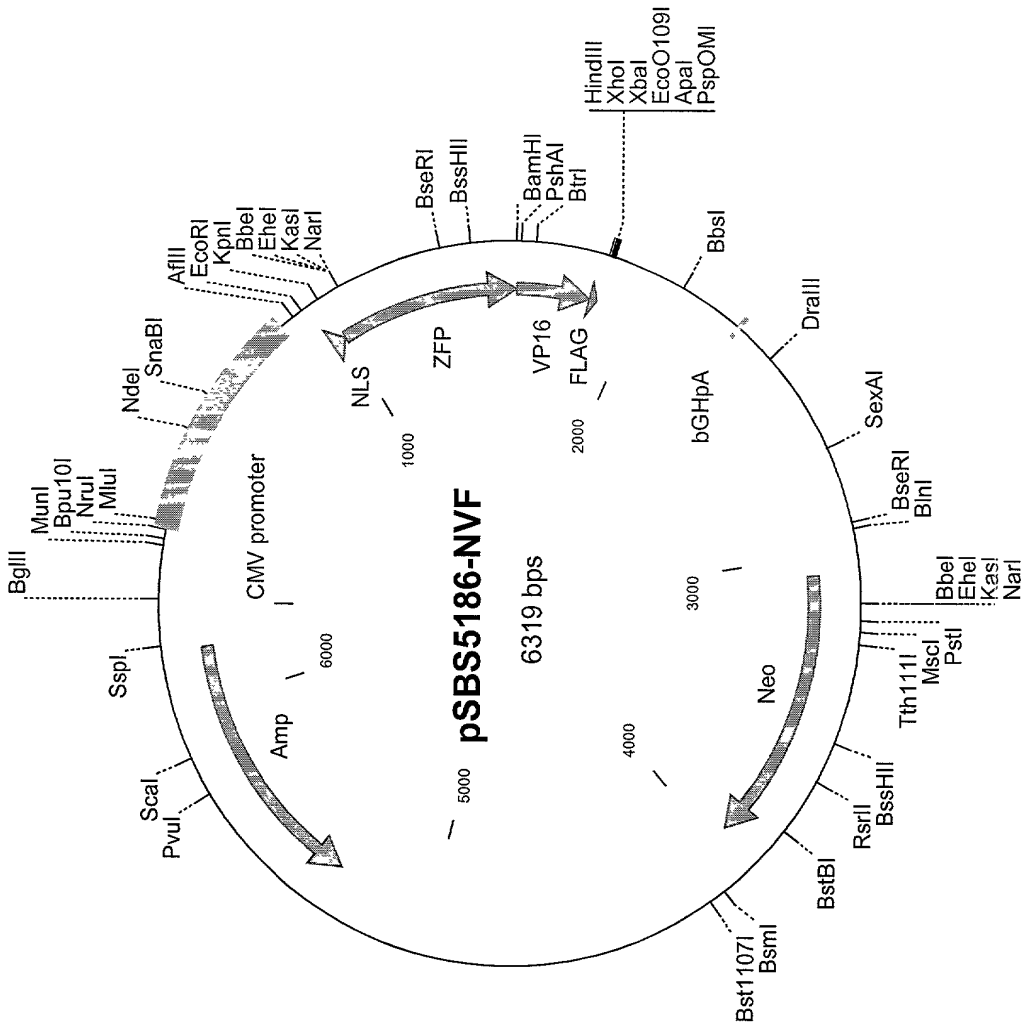
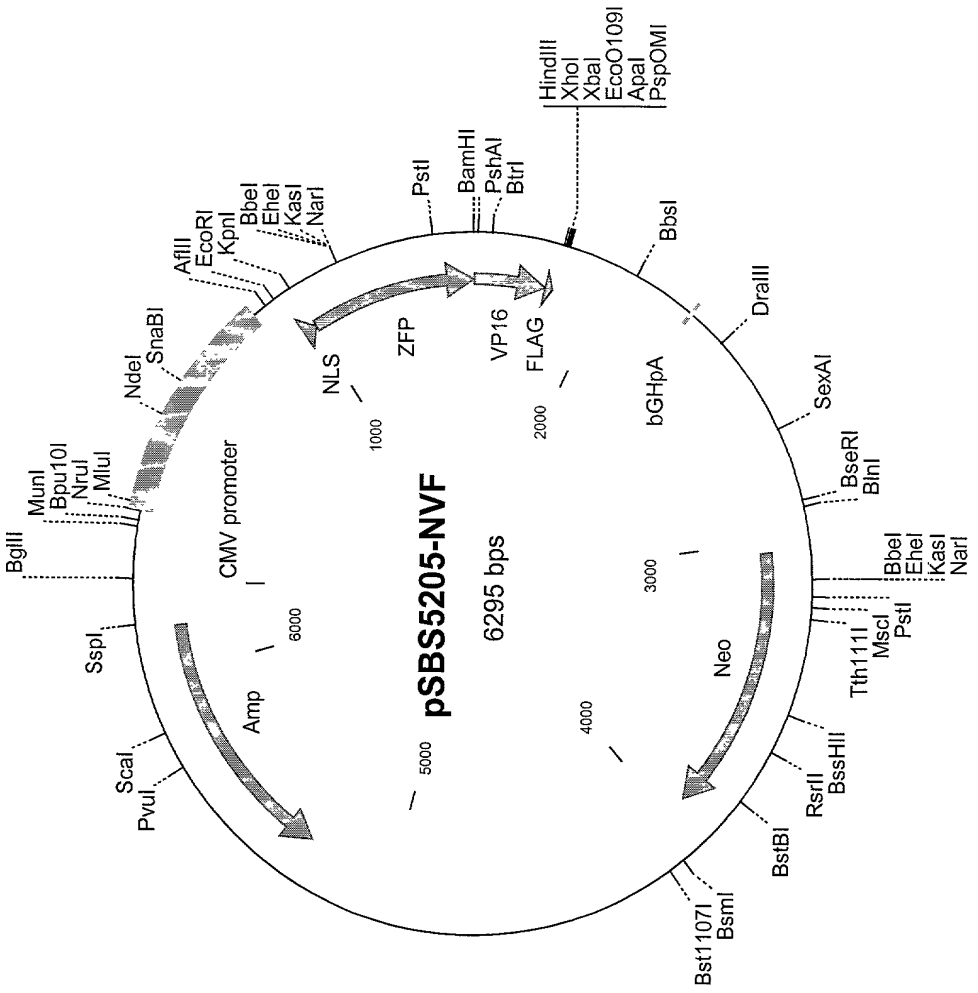

pSBS5186-NVF
6319 bps
CMV promoter
NLS
ZFP
VP16
FLAG
bGHpA
Neo
Amp
1000
2000
3000
4000
5000
6000
HindIII
XhoI
XbaI
EcoO109I
ApaI
PspOMI
BamHI
PshAI
BtrI
BseRI
BssHII
AflII
EcoRI
KpnI
BbeI
EheI
KasI
NarI
NdeI
SnaBI
MunI
Bpu10I
NruI
MluI
BglII
SspI
ScaI
PvuI
Bst1107I
BsmI
BstBI
RsrII
BssHII
Tth111I
MscI
PstI
BbeI
EheI
KasI
NarI
BseRI
BinI
SexAI
DraIII
BbsI

Figure 26D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6295 bps
CMV promoter
NLS
ZFP
VP16
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Neo
Amp
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HindIII
XhoI
XbaI
EcoO109I
ApaI
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DraIII
SexAI
BseRI
BinI
BbeI
EheI
KasI
NarI
MscI
PstI
Tth111I
BssHII
RsrII
BstBI
Bst1107I
BsmI
ScaI
PvuI
SspI
BglII
MunI
Bpu10I
NruI
MluI
NdeI
SnaBI
AflII
EcoRI
KpnI
BbeI
EheI
KasI
NarI
PstI
BamHI
PshAI
BtrI

Figure 26E

```
LOCUS       pSBS5182-N   6319 bp    DNA   CIRCULAR SYN
DEFINITION  Ligation of 5182 into NVF (KpnI, BamHI)
ACCESSION   pSBS5182-N
REFERENCE   1  (bases 1 to 6319)
FEATURES             Location/Qualifiers
     CDS             956..1003
                     /gene="NLS"
                     /product="Nuclear Localization Signal"
     CDS             1004..1597
                     /gene="ZFP"
                     /product="LSR 2B-1A"
     CDS             1598..1840
                     /gene="VP16"
                     /product="VP16 activation domain"
     CDS             1841..1867
                     /gene="FLAG"
                     /product="FLAG epitope"
     CDS             3064..3947
                     /gene="Neo"
                     /product="neomycin resistance"
     CDS             complement (5321..6181)
                     /gene="Amp "
                     /product="Ampcillin resistance"
BASE COUNT     1451 a   1683 c   1651 g   1534 t
ORIGIN
        1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
       61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
      121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
      181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
      241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
      301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
      361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
      421 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
      481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
      541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
      601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
      661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
      721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
      781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
      841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
      901 GTTTAAACTT AAGCTGATCC ACTAGTCCAG TGTGGTGGAA TTCGCTAGCG CCACCATGGC
      961 CCCCAAGAAG AAGAGGAAGG TGGGAATCCA TGGGGTACCG GGCAAGAAGA AGCAGCACAT
     1021 CTGCCACATC CAGGGCTGTG GTAAAGTTTA CGGCGACCGC TCCAACCTGA CCCGCCACCT
     1081 GCGCTGGCAC ACCGGCGAGA GGCCTTTCAT GTGTACATGG TCCTACTGTG GTAAACGCTT
     1141 CACCCAGTCC GGCGACCTGA CCCGCCACAA GCGTACCCAC ACCGGTGAGA AGAAATTTGC
     1201 TTGTCCGGAA TGTCCGAAGC GCTTCATGAT GTCCCACCAC CTGTCCCGCC ACATCAAGAC
     1261 CCACCAGAAC AAGAAGGGTG GATCTGGTGA TGGTGGCCGT CGCGGTGCCG GTTCTGGCAA
     1321 GAAGAAGCAG CACATCTGCC ACATCCAGGG CTGTGGTAAA GTTTACGGCG AGCGCGGCGA
     1381 CCTGACCCGC CACCTGCGCT GGCACACCGG CGAGAGGCCT TTCATGTGTA CATGGTCCTA
     1441 CTGTGGTAAA CGCTTCACCG ACCCGGGCGC CCTGGTGCGC CACAAGCGTA CCCACACCGG
```

Figure 26F

1501 TGAGAAGAAA TTTGCTTGTC CGGAATGTCC GAAGCGCTTC ATGCGCTCCG ACAACCTGAC
1561 CCAGCACATC AAGACCCACC AGAACAAGAA GGGTGGATCC GCCCCCCCGA CCGATGTCAG
1621 CCTGGGGGAC GAGCTCCACT TAGACGGCGA GGACGTGGCG ATGGCGCATG CCGACGCGCT
1681 AGACGATTTC GATCTGGACA TGTTGGGGGA CGGGGATTCC CCGGGGCCGG GATTTACCCC
1741 CCACGACTCC GCCCCCTACG GCGCTCTGGA TATGGCCGGC TTCGAGTTTG AGCAGATGTT
1801 TACCGATGCC CTTGGAATTG ACGAGTACGG TGGGGGCAGC GACTACAAGG ACGACGATGA
1861 CAAGTAAGCT TCTCGAGTCT AGAGGGCCCG TTTAAACCCG CTGATCAGCC TCGACTGTGC
1921 CTTCTAGTTG CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG
1981 GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA
2041 GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG
2101 ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGC TTCTGAGGCG GAAAGAACCA
2161 GCTGGGGCTC TAGGGGGTAT CCCCACGCGC CCTGTAGCGG CGCATTAAGC GCGGCGGGTG
2221 TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC GCTCCTTTCG
2281 CTTTCTTCCC TTCCTTTCTC GCCACGTTCG CCGGCTTTCC CCGTCAAGCT CTAAATCGGG
2341 GCATCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCCAAA AAACTTGATT
2401 AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC GGTTTTTCGC CCTTTGACGT
2461 TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCCAAAC TGGAACAACA CTCAACCCTA
2521 TCTCGGTCTA TTCTTTTGAT TTATAAGGGA TTTTGGGGAT TTCGGCCTAT TGGTTAAAAA
2581 ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTAATTCTG TGGAATGTGT GTCAGTTAGG
2641 GTGTGGAAAG TCCCCAGGCT CCCCAGGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT
2701 AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA
2761 TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA
2821 CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG
2881 AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG
2941 GCCTAGGCTT TTGCAAAAAG CTCCCGGGAG CTTGTATATC CATTTTCGGA TCTGATCAAG
3001 AGACAGGATG AGGATCGTTT CGCATGATTG AACAAGATGG ATTGCACGCA GGTTCTCCGG
3061 CCGCTTGGGT GGAGAGGCTA TTCGGCTATG ACTGGGCACA ACAGACAATC GGCTGCTCTG
3121 ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG GGCGCCCGGT TCTTTTTGTC AAGACCGACC
3181 TGTCCGGTGC CCTGAATGAA CTGCAGGACG AGGCAGCGCG GCTATCGTGG CTGGCCACGA
3241 CGGGCGTTCC TTGCGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAGG GACTGGCTGC
3301 TATTGGGCGA AGTGCCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCCT GCCGAGAAAG
3361 TATCCATCAT GGCTGATGCA ATGCGGCGGC TGCATACGCT TGATCCGGCT ACCTGCCCAT
3421 TCGACCACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATGGAA GCCGGTCTTG
3481 TCGATCAGGA TGATCTGGAC GAAGAGCATC AGGGGCTCGC GCCAGCCGAA CTGTTCGCCA
3541 GGCTCAAGGC GCGCATGCCC GACGGCGAGG ATCTCGTCGT GACCCATGGC GATGCCTGCT
3601 TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCTGGATT CATCGACTGT GGCCGGCTGG
3661 GTGTGGCGGA CCGCTATCAG GACATAGCGT TGGCTACCCG TGATATTGCT GAAGAGCTTG
3721 GCGGCGAATG GGCTGACCGC TTCCTCGTGC TTTACGGTAT CGCCGCTCCC GATTCGCAGC
3781 GCATCGCCTT CTATCGCCTT CTTGACGAGT TCTTCTGAGC GGGACTCTGG GGTTCGAAAT
3841 GACCGACCAA GCGACGCCCA ACCTGCCATC ACGAGATTTC GATTCCACCG CCGCCTTCTA
3901 TGAAAGGTTG GGCTTCGGAA TCGTTTTCCG GGACGCCGGC TGGATGATCC TCCAGCGCGG
3961 GGATCTCATG CTGGAGTTCT TCGCCCACCC CAACTTGTTT ATTGCAGCTT ATAATGGTTA
4021 CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC TGCATTCTAG
4081 TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGTATACCGT CGACCTCTAG
4141 CTAGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC
4201 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT
4261 GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC
4321 GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG
4381 CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT

Figure 26G

```
4441 ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
4501 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC
4561 GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG
4621 GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT
4681 GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG
4741 AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
4801 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG
4861 TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC
4921 TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG
4981 GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT
5041 TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
5101 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC
5161 TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT
5221 GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT
5281 TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG
5341 TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
5401 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC
5461 GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
5521 CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG
5581 GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC
5641 AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
5701 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC
5761 TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
5821 GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC
5881 AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT
5941 ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
6001 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC
6061 TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
6121 AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT
6181 CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG
6241 ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
6301 AAAAGTGCCA CCTGACGTC
//
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Figure 26H

```
LOCUS       pSBS5183-N   6319 bp    DNA   CIRCULAR SYN
DEFINITION  Ligation of 5183 into NVF (KpnI, BamHI)
ACCESSION   pSBS5183-N
REFERENCE   1  (bases 1 to 6319)
FEATURES             Location/Qualifiers
     CDS             956..1003
                     /gene="NLS"
                     /product="Nuclear Localization Signal"
     CDS             1004..1597
                     /gene="ZFP"
                     /product="LSR 4A-3A"
     CDS             1598..1840
                     /gene="VP16"
                     /product="VP16 activation domain"
     CDS             1841..1867
                     /gene="FLAG"
                     /product="FLAG epitope"
     CDS             3064..3947
                     /gene="Neo"
                     /product="neomycin resistance"
     CDS             complement (5321..6181)
                     /gene="Amp "
                     /product="Ampcillin resistance"
BASE COUNT     1446 a   1683 c   1655 g   1535 t
ORIGIN
        1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
       61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
      121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
      181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
      241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
      301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
      361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
      421 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
      481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
      541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
      601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
      661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
      721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
      781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
      841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
      901 GTTTAAACTT AAGCTGATCC ACTAGTCCAG TGTGGTGGAA TTCGCTAGCG CCACCATGGC
      961 CCCCAAGAAG AAGAGGAAGG TGGGAATCCA TGGGGTACCG GGCAAGAAGA AGCAGCACAT
     1021 CTGCCACATC CAGGGCTGTG GTAAAGTTTA CGGCCAGTCC GGCCACCTGG CCCGCCACCT
     1081 GCGCTGGCAC ACCGGCGAGA GGCCTTTCAT GTGTACATGG TCCTACTGTG GTAAACGCTT
     1141 CACCACCTCC GGCGAGCTGG TGCGCCACAA GCGTACCCAC ACCGGTGAGA AGAAATTTGC
     1201 TTGTCCGGAA TGTCCGAAGC GCTTCATGCG TTCCGACCAC CTGTCCCGTC ACATCAAGAC
     1261 CCACCAGAAC AAGAAGGGTG GATCTGGTGA TGGTGGCCGT CGCGGTGGCG GTTCTGGCAA
     1321 GAAGAAGCAG CACATCTGCC ACATCCAGGG CTGTGGTAAA GTTTACGGCG AGCGCGGCGA
     1381 CCTGACCCGC CACCTGCGCT GGCACACCGG CGAGAGGCCT TTCATGTGTA CATGGTCCTA
```

Figure 26I

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1441 CTGTGCTAAA CGCTTCACCC AGCGCGCCCA CCTGGAGCGC CACAAGCGTA CCCACACCGG
1501 TGAGAAGAAA TTTGCTTGTC CGGAATGTCC GAAGCGCTTC ATGCGCTCCG ACGCCCTGAC
1561 CCGCCACATC AAGACCCACC AGAACAAGAA GGGTGGATCC GCCCCCCCGA CCGATGTCAG
1621 CCTGGGGGAC GAGCTCCACT TAGACGGCGA GGACGTGGCG ATGGCGCATG CCGACGCGCT
1681 AGACGATTTC GATCTGGACA TGTTGGGGGA CGGGGATTCC CCGGGGCCGG GATTTACCCC
1741 CCACGACTCC GCCCCCTACG GCGCTCTGGA TATGGCCGGC TTCGAGTTTG AGCAGATGTT
1801 TACCGATGCC CTTGGAATTG ACGAGTACGG TGGGGGCAGC GACTACAAGG ACGACGATGA
1861 CAAGTAAGCT TCTCGAGTCT AGAGGGCCCG TTTAAACCCG CTGATCAGCC TCGACTGTGC
1921 CTTCTAGTTG CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG
1981 GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA
2041 GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG
2101 ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGC TTCTGAGGCG GAAAGAACCA
2161 GCTGGGGCTC TAGGGGGTAT CCCCACGCGC CCTGTAGCGG CGCATTAAGC GCGGCGGGTG
2221 TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC GCTCCTTTCG
2281 CTTTCTTCCC TTCCTTTCTC GCCACGTTCG CCGGCTTTCC CCGTCAAGCT CTAAATCGGG
2341 GCATCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCCAAA AAACTTGATT
2401 AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC GGTTTTTCGC CCTTTGACGT
2461 TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCCAAAC TGGAACAACA CTCAACCCTA
2521 TCTCGGTCTA TTCTTTTGAT TTATAAGGGA TTTTGGGGAT TTCGGCCTAT TGGTTAAAAA
2581 ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTAATTCTG TGGAATGTGT GTCAGTTAGG
2641 GTGTGGAAAG TCCCCAGGCT CCCCAGGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT
2701 AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA
2761 TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA
2821 CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG
2881 AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG
2941 GCCTAGGCTT TTGCAAAAAG CTCCCGGGAG CTTGTATATC CATTTTCGGA TCTGATCAAG
3001 AGACAGGATG AGGATCGTTT CGCATGATTG AACAAGATGG ATTGCACGCA GGTTCTCCGG
3061 CCGCTTGGGT GGAGAGGCTA TTCGGCTATG ACTGGGCACA ACAGACAATC GGCTGCTCTG
3121 ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG GGCGCCCGGT TCTTTTTGTC AAGACCGACC
3181 TGTCCGGTGC CCTGAATGAA CTGCAGGACG AGGCAGCGCG GCTATCGTGG CTGGCCACGA
3241 CGGGCGTTCC TTGCGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAGG GACTGGCTGC
3301 TATTGGGCGA AGTGCCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCCT GCCGAGAAAG
3361 TATCCATCAT GGCTGATGCA ATGCGGCGGC TGCATACGCT TGATCCGGCT ACCTGCCCAT
3421 TCGACCACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATGGAA GCCGGTCTTG
3481 TCGATCAGGA TGATCTGGAC GAAGAGCATC AGGGGCTCGC GCCAGCCGAA CTGTTCGCCA
3541 GGCTCAAGGC GCGCATGCCC GACGGCGAGG ATCTCGTCGT GACCCATGGC GATGCCTGCT
3601 TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCTGGATT CATCGACTGT GGCCGGCTGG
3661 GTGTGGCGGA CCGCTATCAG GACATAGCGT TGGCTACCCG TGATATTGCT GAAGAGCTTG
3721 GCGGCGAATG GGCTGACCGC TTCCTCGTGC TTTACGGTAT CGCCGCTCCC GATTCGCAGC
3781 GCATCGCCTT CTATCGCCTT CTTGACGAGT TCTTCTGAGC GGGACTCTGG GGTTCGAAAT
3841 GACCGACCAA GCGACGCCCA ACCTGCCATC ACGAGATTTC GATTCCACCG CCGCCTTCTA
3901 TGAAAGGTTG GGCTTCGGAA TCGTTTTCCG GGACGCCGGC TGGATGATCC TCCAGCGCGG
3961 GGATCTCATG CTGGAGTTCT TCGCCCACCC CAACTTGTTT ATTGCAGCTT ATAATGGTTA
4021 CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC TGCATTCTAG
4081 TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGTATACCGT CGACCTCTAG
4141 CTAGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC
4201 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT
4261 GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC
4321 GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG
4381 CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT
4441 ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
```

Figure 26J

4501 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC
4561 GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG
4621 GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT
4681 GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG
4741 AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
4801 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG
4861 TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC
4921 TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG
4981 GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT
5041 TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
5101 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC
5161 TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT
5221 GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT
5281 TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG
5341 TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
5401 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC
5461 GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
5521 CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG
5581 GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC
5641 AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
5701 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC
5761 TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
5821 GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC
5881 AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT
5941 ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
6001 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC
6061 TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
6121 AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT
6181 CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG
6241 ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
6301 AAAAGTGCCA CCTGACGTC

Figure 26K

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LOCUS       pSBS5185-N   6295 bp    DNA   CIRCULAR SYN
DEFINITION  Ligation of 5185 into NVF (KpnI, BamHI)
ACCESSION   pSBS5185-N
REFERENCE   1  (bases 1 to 6295)
FEATURES             Location/Qualifiers
     CDS             956..1003
                     /gene="NLS"
                     /product="Nuclear Localization Signal"
     CDS             1004..1573
                     /gene="ZFP"
                     /product="LSR 6A-5A"
     CDS             1574..1816
                     /gene="VP16"
                     /product="VP16 activation domain"
     CDS             1817..1843
                     /gene="FLAG"
                     /product="FLAG epitope"
     CDS             3040..3923
                     /gene="Neo"
                     /product="neomycin resistance"
     CDS             complement (5297..6157)
                     /gene="Amp "
                     /product="Ampcillin resistance"
BASE COUNT     1452 a   1682 c   1635 g   1526 t
ORIGIN
        1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
       61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
      121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
      181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
      241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
      301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
      361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
      421 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
      481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
      541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
      601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
      661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
      721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
      781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
      841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
      901 GTTTAAACTT AAGCTGATCC ACTAGTCCAG TGTGGTGGAA TTCGCTAGCG CCACCATGGC
      961 CCCCAAGAAG AAGAGGAAGG TGGGAATCCA TGGGGTACCG GGCAAGAAGA AGCAGCACAT
     1021 CTGCCACATC CAGGGCTGTG GTAAAGTTTA CGGCCGCTCC GACCACCTGG CCCGCCACCT
     1081 GCGCTGGCAC ACCGGCGAGA GGCCTTTCAT GTGTACATGG TCCTACTGTG GTAAACGCTT
     1141 CACCCGCTCC GACGAGCTGC AGCGCCACAA GCGTACCCAC ACCGGTGAGA AGAAATTTGC
     1201 TTGTCCGGAA TGTCCGAAGC GCTTCATGCG CTCCGACGAG CGCAAGCGCC ACATCAAGAC
     1261 CCACCAGAAC AAGAAGGGTG GATCTGGTGA TGGCAAGAAG AAGCAGCACA TCTGCCACAT
     1321 CCAGGGCTGT GGTAAAGTTT ACGGCCGCTC CGACCACCTG ACCACCCACC TGCGCTGGCA
     1381 CACCGGCGAG AGGCCTTTCA TGTGTACATG GTCCTACTGT GGTAAACGCT TCACCCGCTC
```

Figure 26L

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1441 CGACCACCTG ACCCGCCACA AGCGTACCCA CACCGGTGAG AAGAAATTTG CTTGTCCGGA
1501 ATGTCCGAAG CGCTTCATGC GCTCCGACCA CCTGACCACC CACATCAAGA CCCACCAGAA
1561 CAAGAAGGGT GGATCCGCCC CCCCGACCGA TGTCAGCCTG GGGGACGAGC TCCACTTAGA
1621 CGGCGAGGAC GTGGCGATGG CGCATGCCGA CGCGCTAGAC GATTTCGATC TGGACATGTT
1681 GGGGGACGGG GATTCCCCGG GGCCGGGATT TACCCCCCAC GACTCCGCCC CCTACGGCGC
1741 TCTGGATATG GCCGGCTTCG AGTTTGAGCA GATGTTTACC GATGCCCTTG GAATTGACGA
1801 GTACGGTGGG GGCAGCGACT ACAAGGACGA CGATGACAAG TAAGCTTCTC GAGTCTAGAG
1861 GGCCCGTTTA AACCCGCTGA TCAGCCTCGA CTGTGCCTTC TAGTTGCCAG CCATCTGTTG
1921 TTTGCCCCTC CCCCGTGCCT TCCTTGACCC TGGAAGGTGC CACTCCCACT GTCCTTTCCT
1981 AATAAAATGA GGAAATTGCA TCGCATTGTC TGAGTAGGTG TCATTCTATT CTGGGGGGTG
2041 GGGTGGGGCA GGACAGCAAG GGGGAGGATT GGGAAGACAA TAGCAGGCAT GCTGGGGATG
2101 CGGTGGGCTC TATGGCTTCT GAGGCGGAAA GAACCAGCTG GGGCTCTAGG GGGTATCCCC
2161 ACGCGCCCTG TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT GGTTACGCGC AGCGTGACCG
2221 CTACACTTGC CAGCGCCCTA GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC TTTCTCGCCA
2281 CGTTCGCCGG CTTTCCCCGT CAAGCTCTAA ATCGGGGCAT CCCTTTAGGG TTCCGATTTA
2341 GTGCTTTACG GCACCTCGAC CCCAAAAAAC TTGATTAGGG TGATGGTTCA CGTAGTGGGC
2401 CATCGCCCTG ATAGACGGTT TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG
2461 GACTCTTGTT CCAAACTGGA ACAACACTCA ACCCTATCTC GGTCTATTCT TTTGATTTAT
2521 AAGGGATTTT GGGGATTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA CAAAAATTTA
2581 ACGCGAATTA ATTCTGTGGA ATGTGTGTCA GTTAGGGTGT GGAAAGTCCC CAGGCTCCCC
2641 AGGCAGGCAG AAGTATGCAA AGCATGCATC TCAATTAGTC AGCAACCAGG TGTGGAAAGT
2701 CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCATGCA TCTCAATTAG TCAGCAACCA
2761 TAGTCCCGCC CCTAACTCCG CCCATCCCGC CCCTAACTCC GCCCAGTTCC GCCCATTCTC
2821 CGCCCCATGG CTGACTAATT TTTTTTATTT ATGCAGAGGC CGAGGCCGCC TCTGCCTCTG
2881 AGCTATTCCA GAAGTAGTGA GGAGGCTTTT TTGGAGGCCT AGGCTTTTGC AAAAAGCTCC
2941 CGGGAGCTTG TATATCCATT TTCGGATCTG ATCAAGAGAC AGGATGAGGA TCGTTTCGCA
3001 TGATTGAACA AGATGGATTG CACGCAGGTT CTCCGGCCGC TTGGGTGGAG AGGCTATTCG
3061 GCTATGACTG GGCACAACAG ACAATCGGCT GCTCTGATGC CGCCGTGTTC CGGCTGTCAG
3121 CGCAGGGGCG CCCGGTTCTT TTTGTCAAGA CCGACCTGTC CGGTGCCCTG AATGAACTGC
3181 AGGACGAGGC AGCGCGGCTA TCGTGGCTGG CCACGACGGG CGTTCCTTGC GCAGCTGTGC
3241 TCGACGTTGT CACTGAAGCG GGAAGGGACT GGCTGCTATT GGGCGAAGTG CCGGGGCAGG
3301 ATCTCCTGTC ATCTCACCTT GCTCCTGCCG AGAAAGTATC CATCATGGCT GATGCAATGC
3361 GGCGGCTGCA TACGCTTGAT CCGGCTACCT GCCCATTCGA CCACCAAGCG AAACATCGCA
3421 TCGAGCGAGC ACGTACTCGG ATGGAAGCCG GTCTTGTCGA TCAGGATGAT CTGGACGAAG
3481 AGCATCAGGG GCTCGCGCCA GCCGAACTGT TCGCCAGGCT CAAGGCGCGC ATGCCCGACG
3541 GCGAGGATCT CGTCGTGACC CATGGCGATG CCTGCTTGCC GAATATCATG GTGGAAAATG
3601 GCCGCTTTTC TGGATTCATC GACTGTGGCC GGCTGGGTGT GGCGGACCGC TATCAGGACA
3661 TAGCGTTGGC TACCCGTGAT ATTGCTGAAG AGCTTGGCGG CGAATGGGCT GACCGCTTCC
3721 TCGTGCTTTA CGGTATCGCC GCTCCCGATT CGCAGCGCAT CGCCTTCTAT CGCCTTCTTG
3781 ACGAGTTCTT CTGAGCGGGA CTCTGGGGTT CGAAATGACC GACCAAGCGA CGCCCAACCT
3841 GCCATCACGA GATTTCGATT CCACCGCCGC CTTCTATGAA AGGTTGGGCT TCGGAATCGT
3901 TTTCCGGGAC GCCGGCTGGA TGATCCTCCA GCGCGGGGAT CTCATGCTGG AGTTCTTCGC
3961 CCACCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA
4021 TTTCACAAAT AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA
4081 TGTATCTTAT CATGTCTGTA TACCGTCGAC CTCTAGCTAG AGCTTGGCGT AATCATGGTC
4141 ATAGCTGTTT CCTGTGTGAA ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG
4201 AAGCATAAAG TGTAAAGCCT GGGGTGCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT
4261 GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCGG
4321 CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT CGCTCACTGA
4381 CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT
4441 ACGGTTATCC ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA
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Figure 26M

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4501 AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC
4561 TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA
4621 AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC CGACCCTGCC
4681 GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCAATGCTC
4741 ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA
4801 ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC
4861 GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG
4921 GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT ACACTAGAAG
4981 GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG
5041 CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA
5101 GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA
5161 CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT
5221 CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA GTATATATGA
5281 GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG
5341 TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA
5401 GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC
5461 AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC
5521 TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC
5581 AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC
5641 GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC
5701 CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT
5761 GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC
5821 ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG
5881 TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG
5941 CAGAACTTTA AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT
6001 CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC
6061 ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA
6121 AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT TTCAATATTA
6181 TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA
6241 AAATAAACAA ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTC
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Figure 26N

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LOCUS       pSBS5186-N   6319 bp    DNA   CIRCULAR SYN
DEFINITION  Ligation of 5186 into NVF (KpnI, BamHI)
ACCESSION   pSBS5186-N
REFERENCE   1  (bases 1 to 6319)
FEATURES             Location/Qualifiers
     CDS             956..1003
                     /gene="NLS"
                     /product="Nuclear Localization Signal"
     CDS             1004..1597
                     /gene="ZFP"
                     /product="LSR 8A-7B"
     CDS             1598..1840
                     /gene="VP16"
                     /product="VP16 activation domain"
     CDS             1841..1867
                     /gene="FLAG"
                     /product="FLAG epitope"
     CDS             3064..3947
                     /gene="Neo"
                     /product="neomycin resistance"
     CDS             complement (5321..6181)
                     /gene="Amp "
                     /product="Ampcillin resistance"
BASE COUNT     1449 a   1687 c   1651 g   1532 t
ORIGIN
        1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
       61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
      121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
      181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
      241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
      301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
      361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
      421 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
      481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
      541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
      601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
      661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
      721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
      781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
      841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
      901 GTTTAAACTT AAGCTGATCC ACTAGTCCAG TGTGGTGGAA TTCGCTAGCG CCACCATGGC
      961 CCCCAAGAAG AAGAGGAAGG TGGGAATCCA TGGGGTACCG GGCAAGAAGA AGCAGCACAT
     1021 CTGCCACATC CAGGGCTGTG GTAAAGTTTA CGGCCAGTCC GGCGCCCTGA CCCGCCACCT
     1081 GCGCTGGCAC ACCGGCGAGA GGCCTTTCAT GTGTACATGG TCCTACTGTG GTAAACGCTT
     1141 CACCCGCTCC GACCACCTGA CCCGCCACAA GCGTACCCAC ACCGGTGAGA AGAAATTTGC
     1201 TTGTCCGGAA TGTCCGAAGC GCTTCATGCG CTCCGACAAC CTGCGCGAGC ACAACAAGAC
     1261 CCACCAGAAC AAGAAGGGTG GATCTGGTGA TGGTGGCCGT CGCGGTGGCG GTTCTGGCAA
     1321 GAAGAAGCAG CACATCTGCC ACATCCAGGG CTGTGGTAAA GTTTACGGCC GCTCCTCCGC
```

Figure 26O

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1381 CCTGACCCGC CACCTGCGCT GGCACACCGG CGAGAGGCCT TTCATGTGTA CATGGTCCTA
1441 CTGTGGTAAA CGCTTCACCC AGCGCGCCCA CCTGGAGCGC CACAAGCGTA CCCACACCGG
1501 TGAGAAGAAA TTTGCTTGTC CGGAATGTCC GAAGCGCTTC ATGCGCTCCG ACACCCTGCG
1561 CGAGCACATC AAGACCCACC AGAACAAGAA GGGTGGATCC GCCCCCCCGA CCGATGTCAG
1621 CCTGGGGGAC GAGCTCCACT TAGACGGCGA GGACGTGGCG ATGGCGCATG CCGACGCGCT
1681 AGACGATTTC GATCTGGACA TGTTGGGGGA CGGGGATTCC CCGGGGCCGG GATTTACCCC
1741 CCACGACTCC GCCCCCTACG GCGCTCTGGA TATGGCCGGC TTCGAGTTTG AGCAGATGTT
1801 TACCGATGCC CTTGGAATTG ACGAGTACGG TGGGGGCAGC GACTACAAGG ACGACGATGA
1861 CAAGTAAGCT TCTCGAGTCT AGAGGGCCCG TTTAAACCCG CTGATCAGCC TCGACTGTGC
1921 CTTCTAGTTG CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG
1981 GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA
2041 GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG
2101 ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGC TTCTGAGGCG GAAAGAACCA
2161 GCTGGGGCTC TAGGGGGTAT CCCCACGCGC CCTGTAGCGG CGCATTAAGC GCGGCGGGTG
2221 TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC GCTCCTTTCG
2281 CTTTCTTCCC TTCCTTTCTC GCCACGTTCG CCGGCTTTCC CCGTCAAGCT CTAAATCGGG
2341 GCATCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCCAAA AAACTTGATT
2401 AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC GGTTTTTCGC CCTTTGACGT
2461 TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCCAAAC TGGAACAACA CTCAACCCTA
2521 TCTCGGTCTA TTCTTTTGAT TTATAAGGGA TTTTGGGGAT TTCGGCCTAT TGGTTAAAAA
2581 ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTAATTCTG TGGAATGTGT GTCAGTTAGG
2641 GTGTGGAAAG TCCCCAGGCT CCCCAGGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT
2701 AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA
2761 TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA
2821 CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG
2881 AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG
2941 GCCTAGGCTT TTGCAAAAAG CTCCCGGGAG CTTGTATATC CATTTTCGGA TCTGATCAAG
3001 AGACAGGATG AGGATCGTTT CGCATGATTG AACAAGATGG ATTGCACGCA GGTTCTCCGG
3061 CCGCTTGGGT GGAGAGGCTA TTCGGCTATG ACTGGGCACA ACAGACAATC GGCTGCTCTG
3121 ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG GGCGCCCGGT TCTTTTTGTC AAGACCGACC
3181 TGTCCGGTGC CCTGAATGAA CTGCAGGACG AGGCAGCGCG GCTATCGTGG CTGGCCACGA
3241 CGGGCGTTCC TTGCGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAGG GACTGGCTGC
3301 TATTGGGCGA AGTGCCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCCT GCCGAGAAAG
3361 TATCCATCAT GGCTGATGCA ATGCGGCGGC TGCATACGCT TGATCCGGCT ACCTGCCCAT
3421 TCGACCACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATGGAA GCCGGTCTTG
3481 TCGATCAGGA TGATCTGGAC GAAGAGCATC AGGGGCTCGC GCCAGCCGAA CTGTTCGCCA
3541 GGCTCAAGGC GCGCATGCCC GACGGCGAGG ATCTCGTCGT GACCCATGGC GATGCCTGCT
3601 TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCTGGATT CATCGACTGT GGCCGGCTGG
3661 GTGTGGCGGA CCGCTATCAG GACATAGCGT TGGCTACCCG TGATATTGCT GAAGAGCTTG
3721 GCGGCGAATG GGCTGACCGC TTCCTCGTGC TTTACGGTAT CGCCGCTCCC GATTCGCAGC
3781 GCATCGCCTT CTATCGCCTT CTTGACGAGT TCTTCTGAGC GGGACTCTGG GGTTCGAAAT
3841 GACCGACCAA GCGACGCCCA ACCTGCCATC ACGAGATTTC GATTCCACCG CCGCCTTCTA
3901 TGAAAGGTTG GGCTTCGGAA TCGTTTTCCG GGACGCCGGC TGGATGATCC TCCAGCGCGG
3961 GGATCTCATG CTGGAGTTCT TCGCCCACCC CAACTTGTTT ATTGCAGCTT ATAATGGTTA
4021 CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC TGCATTCTAG
4081 TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGTATACCGT CGACCTCTAG
4141 CTAGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC
4201 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT
4261 GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC
4321 GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG
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Figure 26P

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4381 CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT
4441 ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
4501 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC
4561 GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG
4621 GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT
4681 GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG
4741 AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
4801 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG
4861 TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC
4921 TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG
4981 GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT
5041 TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
5101 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC
5161 TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT
5221 GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT
5281 TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG
5341 TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
5401 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC
5461 GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
5521 CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG
5581 GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC
5641 AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
5701 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC
5761 TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
5821 GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC
5881 AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT
5941 ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
6001 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC
6061 TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
6121 AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT
6181 CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG
6241 ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
6301 AAAAGTGCCA CCTGACGTC
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Figure 26Q

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LOCUS       pSBS5205-N   6295 bp    DNA   CIRCULAR SYN
DEFINITION  Ligation of 5205 into NVF (KpnI, BamHI)
ACCESSION   pSBS5205-N
REFERENCE   1  (bases 1 to 6295)
FEATURES             Location/Qualifiers
     CDS             956..1003
                     /gene="NLS"
                     /product="Nuclear Localization Signal"
     CDS             1004..1573
                     /gene="ZFP"
                     /product="LSR 1A-7B"
     CDS             1574..1816
                     /gene="VP16"
                     /product="VP16 activation domain"
     CDS             1817..1843
                     /gene="FLAG"
                     /product="FLAG epitope"
     CDS             3040..3923
                     /gene="Neo"
                     /product="neomycin resistance"
     CDS             complement (5297..6157)
                     /gene="Amp "
                     /product="Ampcillin resistance"
BASE COUNT     1448 a   1677 c   1643 g   1527 t
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        1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
       61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
      121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
      181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
      241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
      301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
      361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
      421 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
      481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
      541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
      601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
      661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
      721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
      781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
      841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
      901 GTTTAAACTT AAGCTGATCC ACTAGTCCAG TGTGGTGGAA TTCGCTAGCG CCACCATGGC
      961 CCCCAAGAAG AAGAGGAAGG TGGGAATCCA TGGGGTACCG GGCAAGAAGA AGCAGCACAT
     1021 CTGCCACATC CAGGGCTGTG GTAAAGTTTA CGGCGAGCGC GGCGACCTGA CCCGCCACCT
     1081 GCGCTGGCAC ACCGGCGAGA GGCCTTTCAT GTGTACATGG TCCTACTGTG GTAAACGCTT
     1141 CACCGACCCG GGCGCCCTGG TGCGCCACAA GCGTACCCAC ACCGGTGAGA AGAAATTTGC
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     1261 CCACCAGAAC AAGAAGGGTG GATCTGGTGA TGGCAAGAAG AAGCAGCACA TCTGCCACAT
     1321 CCAGGGCTGT GGTAAAGTTT ACGGCCAGTC CGGCACCCTG ACCCGCCACC TGCGCTGGCA
```

Figure 26R

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1501 ATGTCCGAAG CGCTTCATGC GCTCCGACGC CCTGGCCCGC CACATCAAGA CCCACCAGAA
1561 CAAGAAGGGT GGATCCGCCC CCCCGACCGA TGTCAGCCTG GGGGACGAGC TCCACTTAGA
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1681 GGGGGACGGG GATTCCCCGG GGCCGGGATT TACCCCCCAC GACTCCGCCC CCTACGGCGC
1741 TCTGGATATG GCCGGCTTCG AGTTTGAGCA GATGTTTACC GATGCCCTTG GAATTGACGA
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3181 AGGACGAGGC AGCGCGGCTA TCGTGGCTGG CCACGACGGG CGTTCCTTGC GCAGCTGTGC
3241 TCGACGTTGT CACTGAAGCG GGAAGGGACT GGCTGCTATT GGGCGAAGTG CCGGGGCAGG
3301 ATCTCCTGTC ATCTCACCTT GCTCCTGCCG AGAAAGTATC CATCATGGCT GATGCAATGC
3361 GGCGGCTGCA TACGCTTGAT CCGGCTACCT GCCCATTCGA CCACCAAGCG AAACATCGCA
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3481 AGCATCAGGG GCTCGCGCCA GCCGAACTGT TCGCCAGGCT CAAGGCGCGC ATGCCCGACG
3541 GCGAGGATCT CGTCGTGACC CATGGCGATG CCTGCTTGCC GAATATCATG GTGGAAAATG
3601 GCCGCTTTTC TGGATTCATC GACTGTGGCC GGCTGGGTGT GGCGGACCGC TATCAGGACA
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4081 TGTATCTTAT CATGTCTGTA TACCGTCGAC CTCTAGCTAG AGCTTGGCGT AATCATGGTC
4141 ATAGCTGTTT CCTGTGTGAA ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG
4201 AAGCATAAAG TGTAAAGCCT GGGGTGCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT
4261 GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCGG
4321 CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT CGCTCACTGA

Figure 26S

```
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4441 ACGGTTATCC ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA
4501 AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC
4561 TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA
4621 AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC CGACCCTGCC
4681 GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCAATGCTC
4741 ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA
4801 ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC
4861 GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG
4921 GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT ACACTAGAAG
4981 GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG
5041 CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA
5101 GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA
5161 CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT
5221 CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA GTATATATGA
5281 GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG
5341 TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA
5401 GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC
5461 AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC
5521 TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC
5581 AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC
5641 GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC
5701 CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT
5761 GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC
5821 ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG
5881 TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG
5941 CAGAACTTTA AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT
6001 CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC
6061 ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA
6121 AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT TTCAATATTA
6181 TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA
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//
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Figure 26T

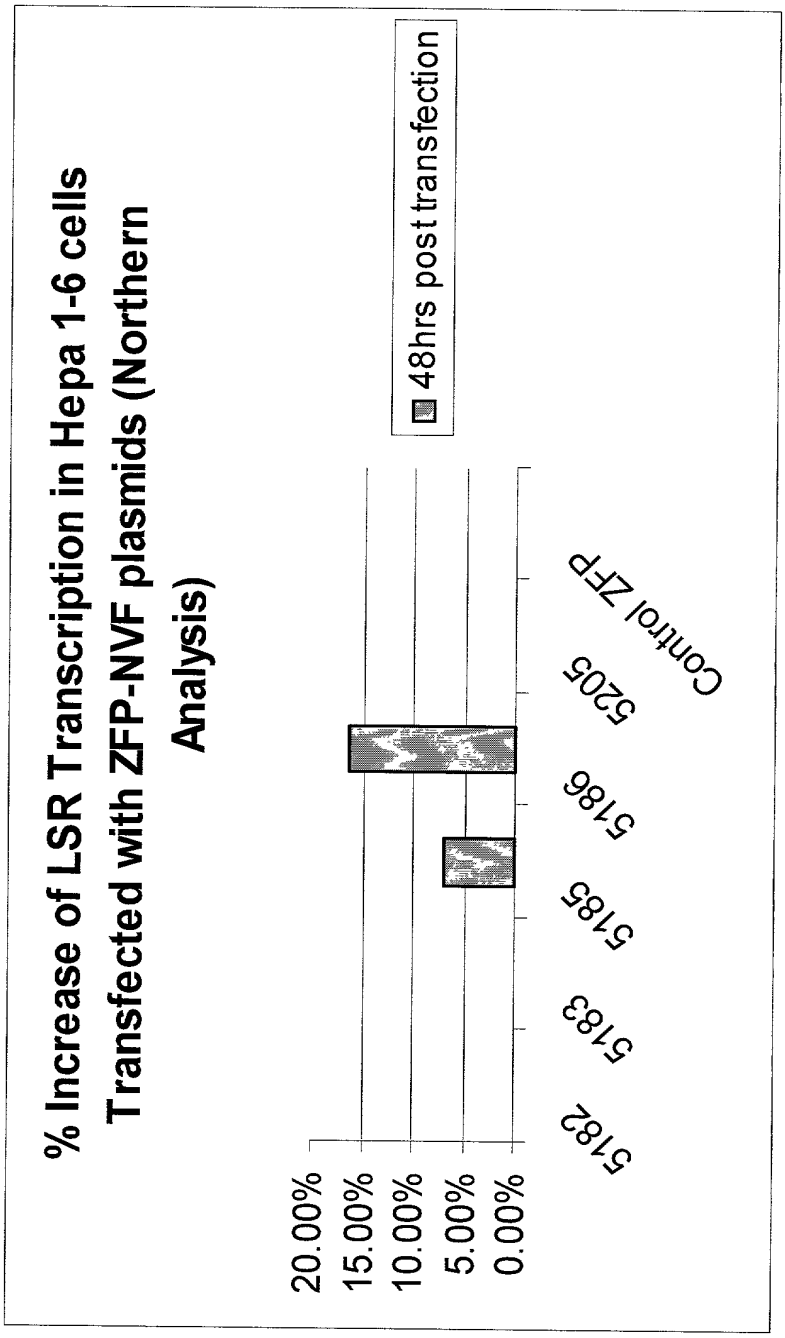

% Increase of LSR Transcription in Hepa 1-6 cells Transfected with ZFP-NVF plasmids (Northern Analysis)
48hrs post transfection
20.00%
15.00%
10.00%
5.00%
0.00%
5182
5183
5185
5186
5205
Control ZFP

Figure 27

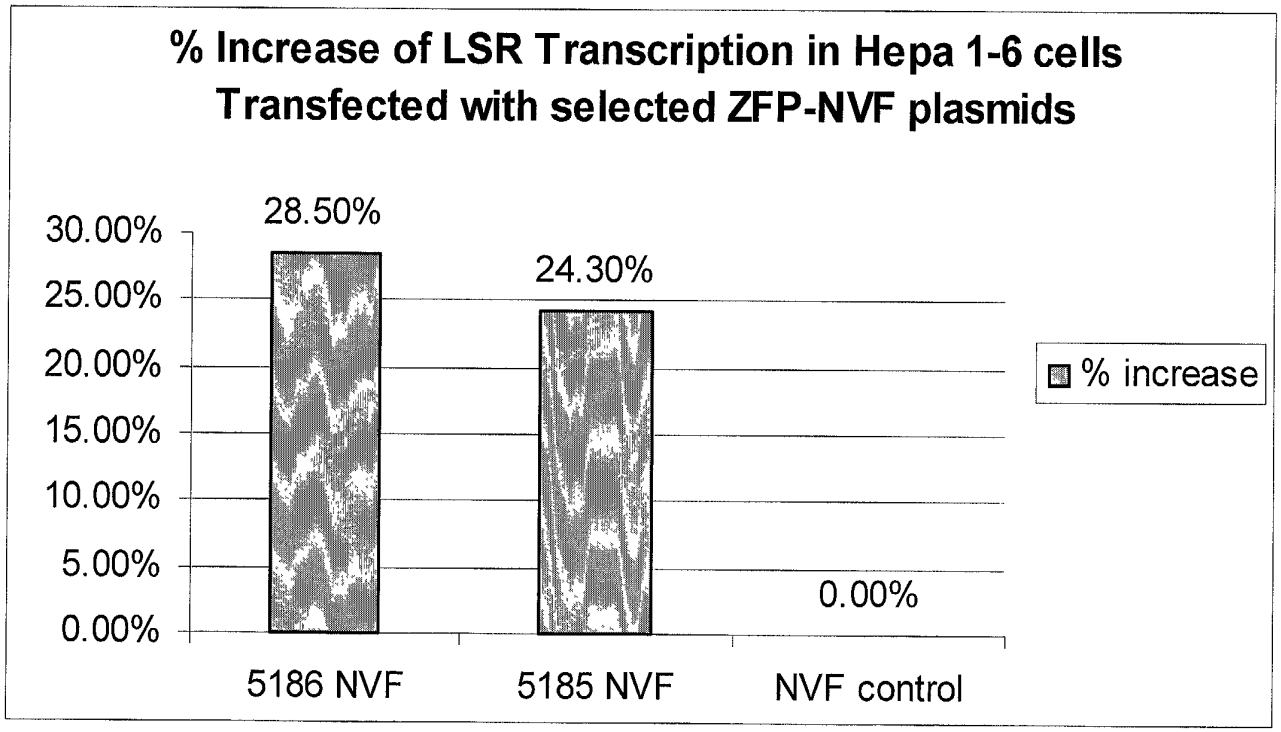

% Increase of LSR Transcription in Hepa 1-6 cells Transfected with selected ZFP-NVF plasmids
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
28.50%
24.30%
0.00%
5186 NVF
5185 NVF
NVF control
% increase

Figure 28

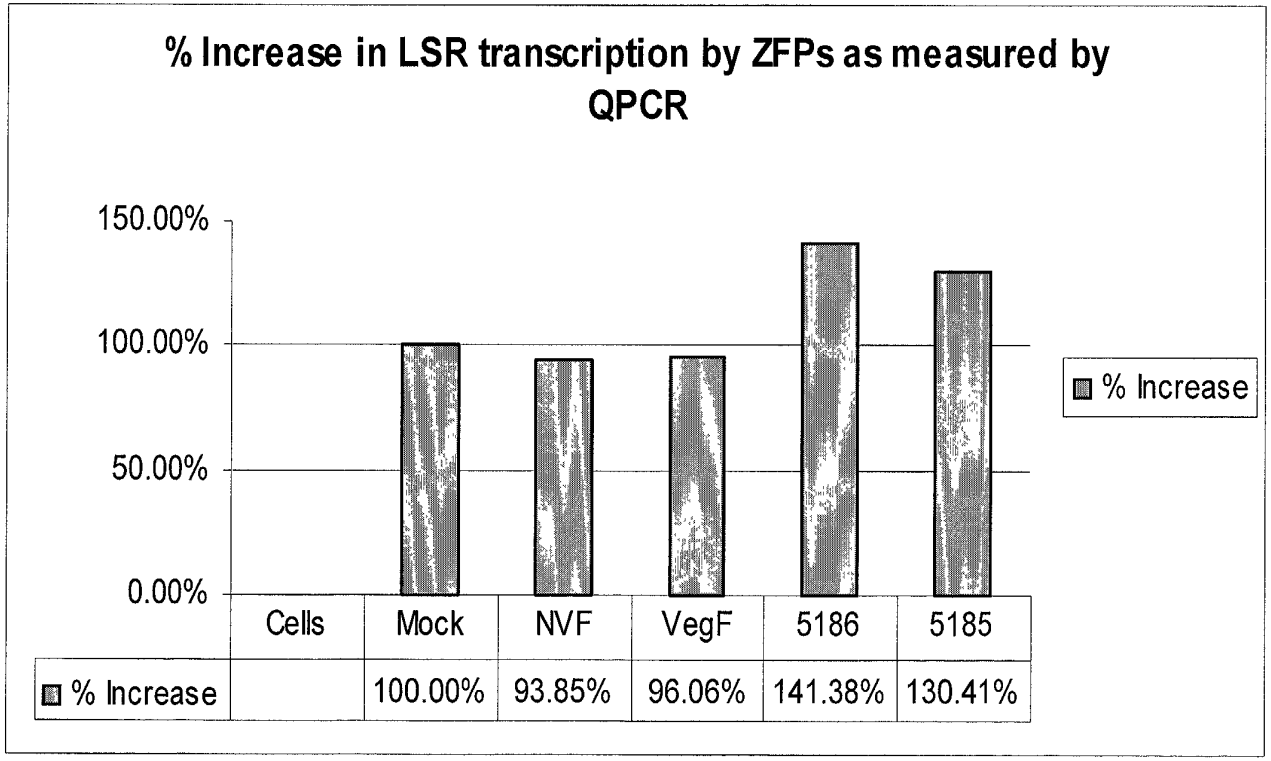
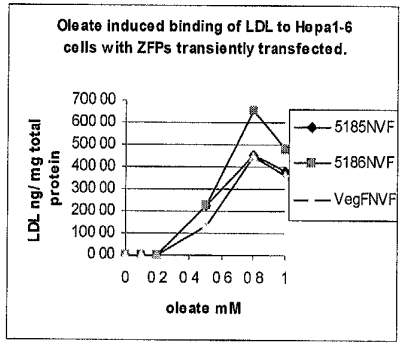

% Increase in LSR transcription by ZFPs as measured by QPCR
150.00%
100.00%
50.00%
0.00%
% Increase
Cells
Mock
NVF
VegF
5186
5185
% Increase
100.00%
93.85%
96.06%
141.38%
130.41%

Figure 29

30.A



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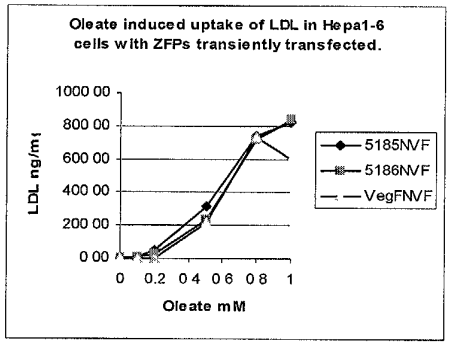

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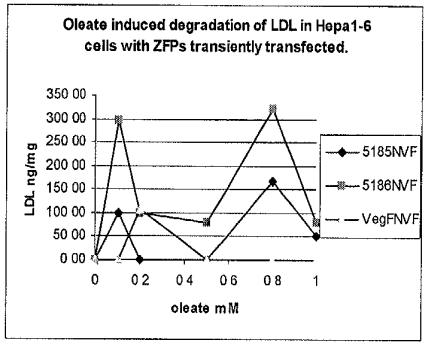

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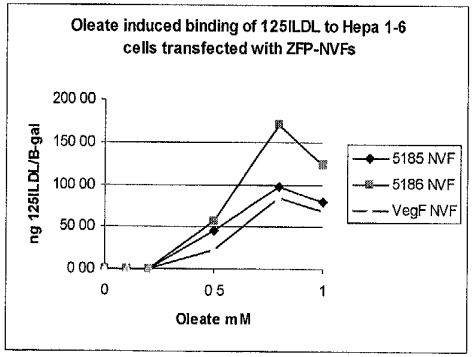

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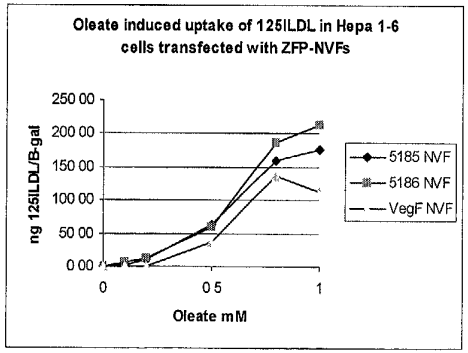

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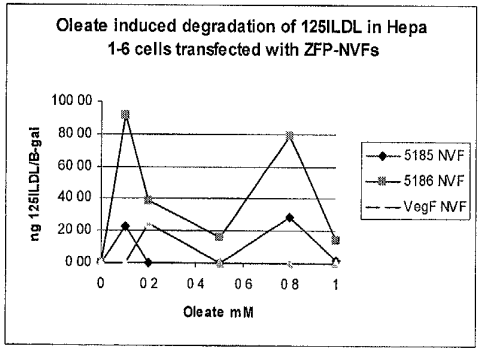

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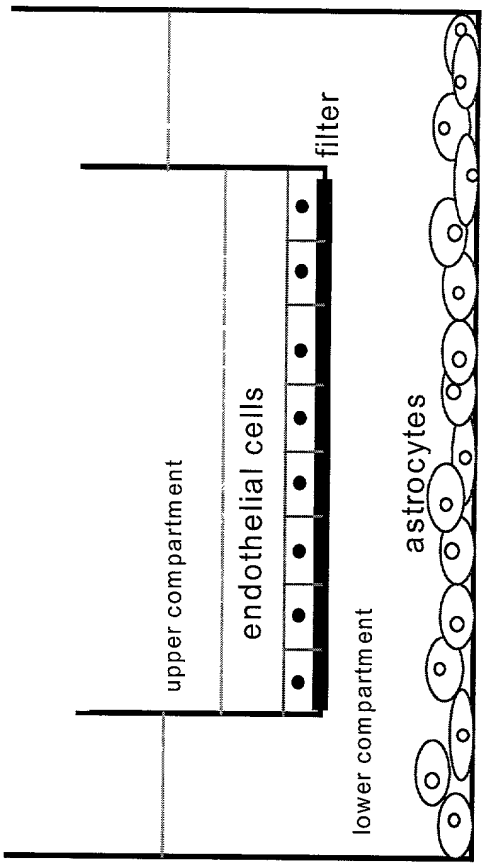

upper compartment
endothelial cells
filter
lower compartment
astrocytes

Figure 31

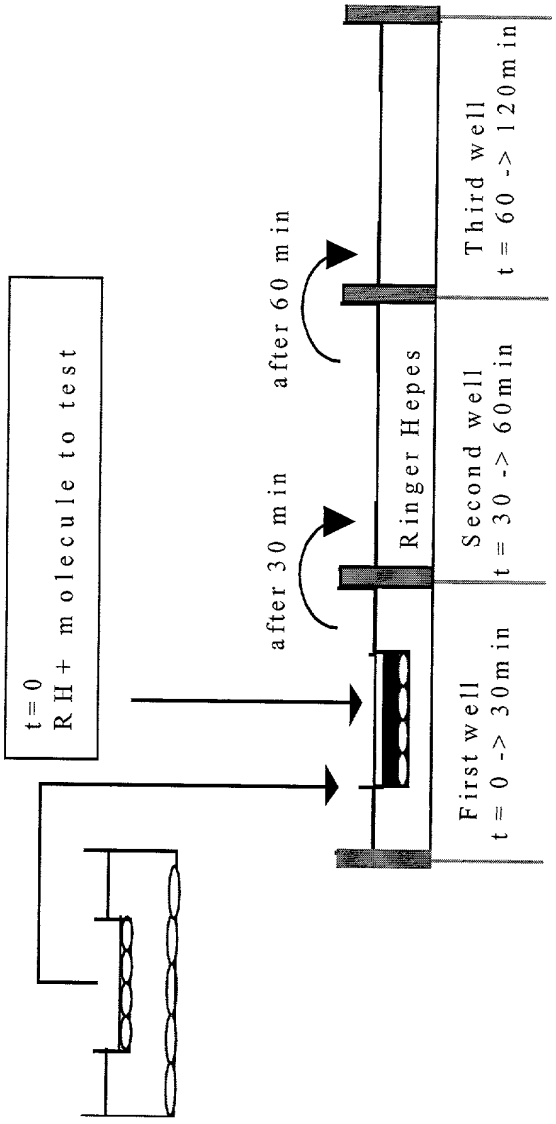

t = 0
RH + molecule to test
after 30 min
after 60 min
Ringer Hepes
First well
t = 0 -> 30 min
Second well
t = 30 -> 60 min
Third well
t = 60 -> 120 min

Figure 32

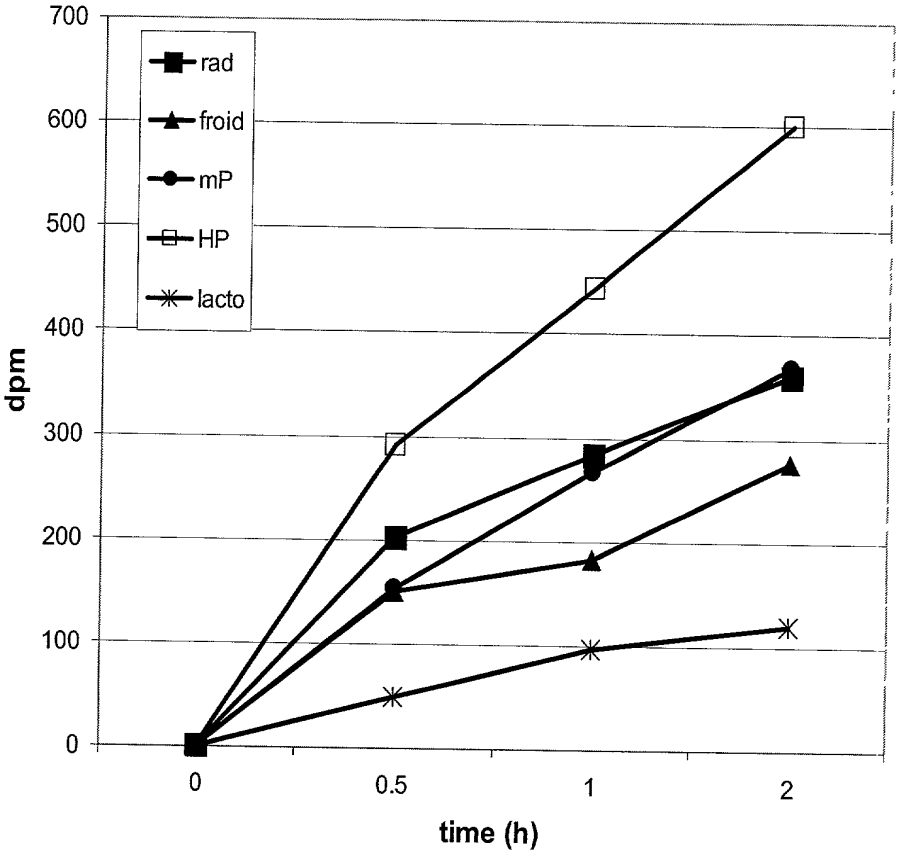
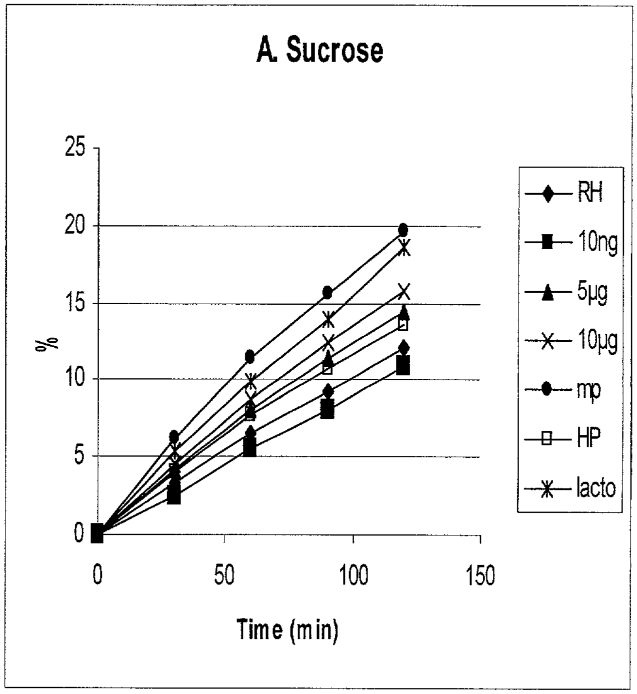

700
600
500
400
300
200
100
0
dpm
rad
froid
mP
HP
lacto
0
0.5
1
2
time (h)

Figure 33

34A



34B

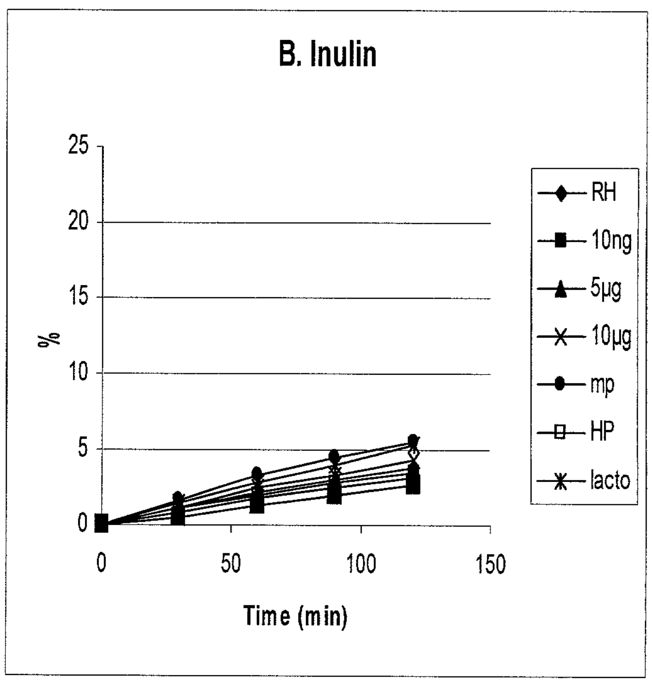
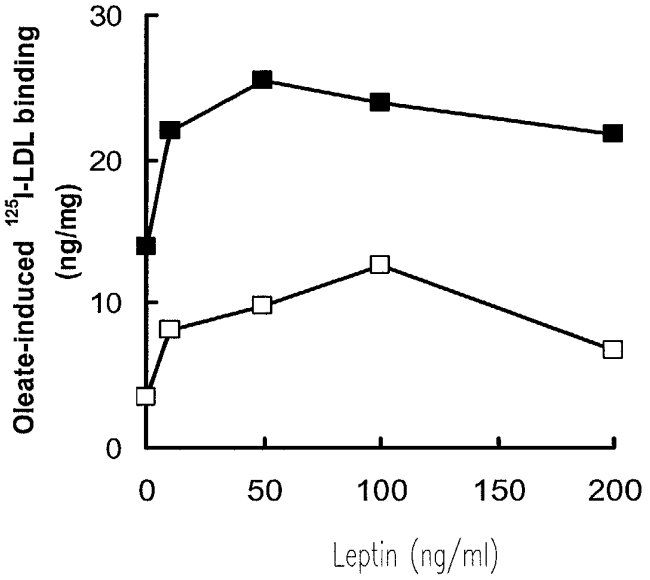


Figure 34

35A



35B

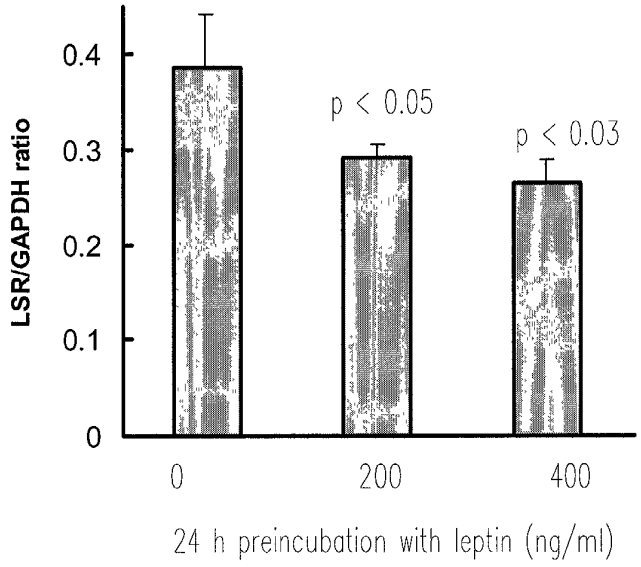


Figure 35

PATENT
70.US2.REG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Yen, Frances, et al
Appl. No.	:	To be assigned
Filed	:	Herewith
For	:	METHODS OF SCREENING FOR COMPOUNDS THAT MODULATE THE LSR-LEPTIN INTERACTION AND THEIR USE IN THE PREVENTION AND TREATMENT OF OBESITY-RELATED DISEASES

SEQUENCE SUBMISSION STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

A copy of the Sequence Listing in computer readable form as required by 37 C.F.R. § 1.821(e) is submitted herewith.

As required by 37 C.F.R. § 1.821(f), the data on the enclosed disk is identical to the Sequence Listing in the application filed herewith.

Respectfully submitted,

Dated: 9-22-00

By: tde Langen

Tia E. de Langen
Paralegal
Genset Corporation
875 Prospect Street
Suite #206
La Jolla, CA 92037
(858) 551-3000

SEQUENCE LISTING

<110> Yen, Frances
Erickson, Mary Ruth
Fruebis, Joachim
Bihain, Bernard

<120> Methods Of Screening For Compounds That Modulate the LSR-Leptin Interaction and Their Use in the Prevention and Treatment of Obesity-Related Diseases

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gga ctt gga gta ggg aca agg aac gga agt ggg aag ggg agg agc gtg       163
Gly Leu Gly Val Gly Thr Arg Asn Gly Ser Gly Lys Gly Arg Ser Val
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cac ccc tcc tgg cct tgg tgc gcg ccg cgc ccc cta agg tac ttt gga       211
His Pro Ser Trp Pro Trp Cys Ala Pro Arg Pro Leu Arg Tyr Phe Gly
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agg gac gcg cgg gcc aga cgc gcc cag acg gcc gcg atg gcg ctg ttg       259
Arg Asp Ala Arg Ala Arg Arg Ala Gln Thr Ala Ala Met Ala Leu Leu
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gcc ggc ggg ctc tcc aga ggg ctg ggc tcc cac ccg gcc gcc gca ggc       307
Ala Gly Gly Leu Ser Arg Gly Leu Gly Ser His Pro Ala Ala Ala Gly
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Arg Asp Ala Val Val Phe Val Trp Leu Leu Leu Ser Thr Trp Cys Thr
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gct cct gcc agg gcc atc cag gtg acc gtg tcc aac ccc tac cac gtg       403
Ala Pro Ala Arg Ala Ile Gln Val Thr Val Ser Asn Pro Tyr His Val
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gtg atc ctc ttc cag cct gtg acc ctg ccc tgt acc tac cag atg acc       451
Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Thr
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tcg acc ccc acg caa ccc atc gtc atc tgg aag tac aag tct ttc tgc       499
Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys
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cgg gac cgc atc gcc gat gcc ttc tcc ccg gcc agc gtc gac aac cag       547
Arg Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln
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ctc aat gcc cag ctg gca gcc ggg aac cca ggc tac aac ccc tac gty       595
Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val
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gag tgc cag gac agc gtg cgc acc gtc agg gtc gtg gcc acc aag cag       643
Glu Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln
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ggc aac gct gtg acc ctg gga gat tac tac cag ggc cgg agg att acc       691
Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr
                185                 190                 195
atc acc gga aat gct gac ctg acc ttt gac cag acg gcg tgg ggg gac       739
Ile Thr Gly Asn Ala Asp Leu Thr Phe Asp Gln Thr Ala Trp Gly Asp
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agt ggt gtg tat tac tgc tcc gtg gtc tca gcc cag gac ctc cag ggg       787
Ser Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Gln Gly
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aac aat gag gcc tac gca gag ctc atc gtc ctt ggg agg acc tca ggg       835
Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Gly Arg Thr Ser Gly
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gtg gct gag ctc tta cct ggt ttt cag gcg ggg ccc ata gaa gac tgg       883
Val Ala Glu Leu Leu Pro Gly Phe Gln Ala Gly Pro Ile Glu Asp Trp
245                 250                 255                 260
ctc ttc gtg gtt gtg gta tgc ctg gct gcc ttc ctc atc ttc ctc ctc       931
Leu Phe Val Val Val Val Cys Leu Ala Ala Phe Leu Ile Phe Leu Leu
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ctg ggc aty tgc tgg tgc cag tgc tgc ccg cac act tgc tgc tgc tac       979
Leu Gly Ile Cys Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr
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gtc agg tgc ccc tgc tgc cca gac aag tgc tgc tgc ccc gag gcc ctg      1027
Val Arg Cys Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu
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tat gcc gcc ggc aaa gca gcc acc tca ggt gtt ccc agc att tat gcc      1075
Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala
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gct atg att ccc atg ggc cct gcc tac aac ggg tac cct gga gga tac     1171
Ala Met Ile Pro Met Gly Pro Ala Tyr Asn Gly Tyr Pro Gly Gly Tyr
                345                 350                 355
cct gga gac gtt gac agg art agc tca gct ggt ggc caa ggc tcc tat     1219
Pro Gly Asp Val Asp Arg Xaa Ser Ser Ala Gly Gly Gln Gly Ser Tyr
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gta ccc ctg ctt cgg gac acg gac agc agt gtg gcc tct gaa gtc cgc     1267
Val Pro Leu Leu Arg Asp Thr Asp Ser Ser Val Ala Ser Glu Val Arg
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agt ggc tac agg att cag gcc agc cag cag gac gac tcc atg cgg gtc     1315
Ser Gly Tyr Arg Ile Gln Ala Ser Gln Gln Asp Asp Ser Met Arg Val
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ctg tac tac atg gag aag gag ctg gcc aac ttc gac cct tct cga cst     1363
Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Xaa
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ggc ccc ccc agt ggc cgt gtg gag cgg gcc atg agt gaa gtc acc tcc     1411
Gly Pro Pro Ser Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser
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ctc cac gag gac gac tgg cga tct cgg cct tcc cgg ggc cct gcc ctc     1459
Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Gly Pro Ala Leu
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acc ccg atc cgg gat gag gag tgg ggt ggc cac tcc ccc cgg agt ccc     1507
Thr Pro Ile Arg Asp Glu Glu Trp Gly Gly His Ser Pro Arg Ser Pro
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agg gga tgg gac cag gag ccc gcc agg gag cag gca ggc ggg ggc tgg     1555
Arg Gly Trp Asp Gln Glu Pro Ala Arg Glu Gln Ala Gly Gly Gly Trp
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cgg gcc agg cgg ccc cgg gcc cgc tcc gtg gac gcc ctg gac gac ctc     1603
Arg Ala Arg Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Leu
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acc ccg ccg agc acc gcc gag tca ggg agc agg tct ccc acg agt aat     1651
Thr Pro Pro Ser Thr Ala Glu Ser Gly Ser Arg Ser Pro Thr Ser Asn
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ggt ggg aga agc cgg gcc tac atg ccc ccg cgg agc cgc agc cgg gac     1699
Gly Gly Arg Ser Arg Ala Tyr Met Pro Pro Arg Ser Arg Ser Arg Asp
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gac ctc tat gac caa gac gac tcg agg gac ttc cca cgc tcc cgg gac     1747
Asp Leu Tyr Asp Gln Asp Asp Ser Arg Asp Phe Pro Arg Ser Arg Asp
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ccc cac tac gac gac ttc agg tct cgg gag cgc cct cct gcc gac ccc     1795
Pro His Tyr Asp Asp Phe Arg Ser Arg Glu Arg Pro Pro Ala Asp Pro
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agg tcc cac cac cac cgt acc cgg gac cct cgg gac aac ggc tcc agg     1843
Arg Ser His His His Arg Thr Arg Asp Pro Arg Asp Asn Gly Ser Arg
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tcc ggg gac ctc ccc tat gat ggg cgg cta ctg gag gag gct gtg agg     1891
Ser Gly Asp Leu Pro Tyr Asp Gly Arg Leu Leu Glu Glu Ala Val Arg
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aag aag ggg tcg gag gag agg agg aga ccc cac aag gag gag gag gaa     1939
Lys Lys Gly Ser Glu Glu Arg Arg Arg Pro His Lys Glu Glu Glu Glu
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gag gcc tac tac ccg ccc gcg ccg ccc ccg tac tcg gag acc gac tcg     1987
Glu Ala Tyr Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser
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cag gcg tcc cga gag cgc agg ctc aag aag aac ttg gcc ctg agt cgg     2035
Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser Arg
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gaa agt tta gtc gtc tga tctgacgttt tctacgtagc ttttgkattt            2083
Glu Ser Leu Val Val  *
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Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr
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Tyr Gln Met Thr Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr
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<221> allele
<222> 595
<223> 9-3-324 : polymorphic base C or T

<220>
<221> allele
<222> 883
<223> 9-6-187 : polymorphic base C or T

<220>
<221> allele
<222> 1134

<223> 9-7-325 : polymorphic base A or G

<220>
<221> allele
<222> 1305
<223> 9-9-246 : polymorphic base G or C

<220>
<221> allele
<222> 1601
<223> LSRX9f13-BM : polymorphic base deletion of AGG

<220>
<221> allele
<222> 2022
<223> LSRX9f14-BM : polymorphic base T or G

<400> 4

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tggagtgtgg ctcggaggac cgcggcgggt caagcacctt tctcccccat atctgaaagc      60
atgccctttg tccacgtcgt ttacgctcat taaaacttcc aga atg caa cag gac      115
                                                 Met Gln Gln Asp
                                                 1
gga ctt gga gta ggg aca agg aac gga agt ggg aag ggg agg agc gtg      163
Gly Leu Gly Val Gly Thr Arg Asn Gly Ser Gly Lys Gly Arg Ser Val
5                   10                  15                  20
cac ccc tcc tgg cct tgg tgc gcg ccg cgc ccc cta agg tac ttt gga      211
His Pro Ser Trp Pro Trp Cys Ala Pro Arg Pro Leu Arg Tyr Phe Gly
                25                  30                  35
agg gac gcg cgg gcc aga cgc gcc cag acg gcc gcg atg gcg ctg ttg      259
Arg Asp Ala Arg Ala Arg Arg Ala Gln Thr Ala Ala Met Ala Leu Leu
            40                  45                  50
gcc ggc ggg ctc tcc aga ggg ctg ggc tcc cac ccg gcc gcc gca ggc      307
Ala Gly Gly Leu Ser Arg Gly Leu Gly Ser His Pro Ala Ala Ala Gly
        55                  60                  65
cgg gac gcg gtc gtc ttc gtg tgg ctt ctg ctt agc acc tgg tgc aca      355
Arg Asp Ala Val Val Phe Val Trp Leu Leu Leu Ser Thr Trp Cys Thr
    70                  75                  80
gct cct gcc agg gcc atc cag gtg acc gtg tcc aac ccc tac cac gtg      403
Ala Pro Ala Arg Ala Ile Gln Val Thr Val Ser Asn Pro Tyr His Val
85                  90                  95                  100
gtg atc ctc ttc cag cct gtg acc ctg ccc tgt acc tac cag atg acc      451
Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Thr
                105                 110                 115
tcg acc ccc acg caa ccc atc gtc atc tgg aag tac aag tct ttc tgc      499
Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys
            120                 125                 130
cgg gac cgc atc gcc gat gcc ttc tcc ccg gcc agc gtc gac aac cag      547
Arg Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln
        135                 140                 145
ctc aat gcc cag ctg gca gcc ggg aac cca ggc tac aac ccc tac gty      595
Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val
    150                 155                 160
gag tgc cag gac agc gtg cgc acc gtc agg gtc gtg gcc acc aag cag      643
Glu Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln
165                 170                 175                 180
ggc aac gct gtg acc ctg gga gat tac tac cag ggc cgg agg att acc      691
Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr
                185                 190                 195
atc acc gga aat gct gac ctg acc ttt gac cag acg gcg tgg ggg gac      739
Ile Thr Gly Asn Ala Asp Leu Thr Phe Asp Gln Thr Ala Trp Gly Asp
            200                 205                 210
agt ggt gtg tat tac tgc tcc gtg gtc tca gcc cag gac ctc cag ggg      787
Ser Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Gln Gly
```

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        215                 220                 225
aac aat gag gcc tac gca gag ctc atc gtc ctt gac tgg ctc ttc gtg      835
Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Asp Trp Leu Phe Val
    230                 235                 240
gtt gtg gta tgc ctg gct gcc ttc ctc atc ttc ctc ctc ctg ggc aty      883
Val Val Val Cys Leu Ala Ala Phe Leu Ile Phe Leu Leu Leu Gly Ile
245                 250                 255                 260
tgc tgg tgc cag tgc tgc ccg cac act tgc tgc tgc tac gtc agg tgc      931
Cys Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr Val Arg Cys
                265                 270                 275
ccc tgc tgc cca gac aag tgc tgc tgc ccc gag gcc ctg tat gcc gcc      979
Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr Ala Ala
            280                 285                 290
ggc aaa gca gcc acc tca ggt gtt ccc agc att tat gcc ccc agc acc     1027
Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Thr
        295                 300                 305
tat gcc cac ctg tct ccc gcc aag acc cca ccc cca cca gct atg att     1075
Tyr Ala His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Ala Met Ile
    310                 315                 320
ccc atg ggc cct gcc tac aac ggg tac cct gga gga tac cct gga gac     1123
Pro Met Gly Pro Ala Tyr Asn Gly Tyr Pro Gly Gly Tyr Pro Gly Asp
325                 330                 335                 340
gtt gac agg art agc tca gct ggt ggc caa ggc tcc tat gta ccc ctg     1171
Val Asp Arg Xaa Ser Ser Ala Gly Gly Gln Gly Ser Tyr Val Pro Leu
                345                 350                 355
ctt cgg gac acg gac agc agt gtg gcc tct gaa gtc cgc agt ggc tac     1219
Leu Arg Asp Thr Asp Ser Ser Val Ala Ser Glu Val Arg Ser Gly Tyr
            360                 365                 370
agg att cag gcc agc cag cag gac gac tcc atg cgg gtc ctg tac tac     1267
Arg Ile Gln Ala Ser Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr
        375                 380                 385
atg gag aag gag ctg gcc aac ttc gac cct tct cga cst ggc ccc ccc     1315
Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Xaa Gly Pro Pro
    390                 395                 400
agt ggc cgt gtg gag cgg gcc atg agt gaa gtc acc tcc ctc cac gag     1363
Ser Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu His Glu
405                 410                 415                 420
gac gac tgg cga tct cgg cct tcc cgg ggc cct gcc ctc acc ccg atc     1411
Asp Asp Trp Arg Ser Arg Pro Ser Arg Gly Pro Ala Leu Thr Pro Ile
                425                 430                 435
cgg gat gag gag tgg ggt ggc cac tcc ccc cgg agt ccc agg gga tgg     1459
Arg Asp Glu Glu Trp Gly Gly His Ser Pro Arg Ser Pro Arg Gly Trp
            440                 445                 450
gac cag gag ccc gcc agg gag cag gca ggc ggg ggc tgg cgg gcc agg     1507
Asp Gln Glu Pro Ala Arg Glu Gln Ala Gly Gly Gly Trp Arg Ala Arg
        455                 460                 465
cgg ccc cgg gcc cgc tcc gtg gac gcc ctg gac gac ctc acc ccg ccg     1555
Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Leu Thr Pro Pro
    470                 475                 480
agc acc gcc gag tca ggg agc agg tct ccc acg agt aat ggt ggg aga     1603
Ser Thr Ala Glu Ser Gly Ser Arg Ser Pro Thr Ser Asn Gly Gly Arg
485                 490                 495                 500
agc cgg gcc tac atg ccc ccg cgg agc cgc agc cgg gac gac ctc tat     1651
Ser Arg Ala Tyr Met Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr
                505                 510                 515
gac caa gac gac tcg agg gac ttc cca cgc tcc cgg gac ccc cac tac     1699
Asp Gln Asp Asp Ser Arg Asp Phe Pro Arg Ser Arg Asp Pro His Tyr
            520                 525                 530
gac gac ttc agg tct cgg gag cgc cct cct gcc gac ccc agg tcc cac     1747
Asp Asp Phe Arg Ser Arg Glu Arg Pro Pro Ala Asp Pro Arg Ser His
        535                 540                 545
cac cac cgt acc cgg gac cct cgg gac aac ggc tcc agg tcc ggg gac     1795
His His Arg Thr Arg Asp Pro Arg Asp Asn Gly Ser Arg Ser Gly Asp
```

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    550                 555                 560
ctc ccc tat gat ggg cgg cta ctg gag gag gct gtg agg aag aag ggg     1843
Leu Pro Tyr Asp Gly Arg Leu Leu Glu Glu Ala Val Arg Lys Lys Gly
565                 570                 575                 580
tcg gag gag agg agg aga ccc cac aag gag gag gag gaa gag gcc tac     1891
Ser Glu Glu Arg Arg Arg Pro His Lys Glu Glu Glu Glu Glu Ala Tyr
                585                 590                 595
tac ccg ccc gcg ccg ccc ccg tac tcg gag acc gac tcg cag gcg tcc     1939
Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser
            600                 605                 610
cga gag cgc agg ctc aag aag aac ttg gcc ctg agt cgg gaa agt tta     1987
Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu
        615                 620                 625
gtc gtc tga tctgacgttt tctacgtagc ttttgkattt tttttttaa              2036
Val Val  *
    630
tttgaaggaa cactgatgaa gccctgccat acccctcccg agtctaataa aacgtataat   2096
cacaa                                                               2101
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<210> 5
<211> 630
<212> PRT
<213> Homo sapiens

<220>
<221> VARIANT
<222> 344
<223> 9-7-325 : polymorphic amino acid Ser or Asn

<220>
<221> VARIANT
<222> 401
<223> 9-9-246 : polymorphic amino acid Pro or Arg

<220>
<221> VARIANT
<222> 500
<223> LSRX9f13-BM : polymorphic amino acid deletion of Arg

<400> 5

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Met Gln Gln Asp Gly Leu Gly Val Gly Thr Arg Asn Gly Ser Gly Lys
1               5                  10                  15
Gly Arg Ser Val His Pro Ser Trp Pro Trp Cys Ala Pro Arg Pro Leu
            20                  25                  30
Arg Tyr Phe Gly Arg Asp Ala Arg Ala Arg Arg Ala Gln Thr Ala Ala
        35                  40                  45
Met Ala Leu Leu Ala Gly Gly Leu Ser Arg Gly Leu Gly Ser His Pro
    50                  55                  60
Ala Ala Ala Gly Arg Asp Ala Val Val Phe Val Trp Leu Leu Leu Ser
65                  70                  75                  80
Thr Trp Cys Thr Ala Pro Ala Arg Ala Ile Gln Val Thr Val Ser Asn
                85                  90                  95
Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr
            100                 105                 110
Tyr Gln Met Thr Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr
        115                 120                 125
Lys Ser Phe Cys Arg Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser
    130                 135                 140
Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr
145                 150                 155                 160
Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg Thr Val Arg Val Val
                165                 170                 175
Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly
```

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            180                 185                 190
Arg Arg Ile Thr Ile Thr Gly Asn Ala Asp Leu Thr Phe Asp Gln Thr
        195                 200                 205
Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln
    210                 215                 220
Asp Leu Gln Gly Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Asp
225                 230                 235                 240
Trp Leu Phe Val Val Val Val Cys Leu Ala Ala Phe Leu Ile Phe Leu
                245                 250                 255
Leu Leu Gly Ile Cys Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys
            260                 265                 270
Tyr Val Arg Cys Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala
        275                 280                 285
Leu Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr
    290                 295                 300
Ala Pro Ser Thr Tyr Ala His Leu Ser Pro Ala Lys Thr Pro Pro Pro
305                 310                 315                 320
Pro Ala Met Ile Pro Met Gly Pro Ala Tyr Asn Gly Tyr Pro Gly Gly
                325                 330                 335
Tyr Pro Gly Asp Val Asp Arg Ser Ser Ser Ala Gly Gly Gln Gly Ser
            340                 345                 350
Tyr Val Pro Leu Leu Arg Asp Thr Asp Ser Ser Val Ala Ser Glu Val
        355                 360                 365
Arg Ser Gly Tyr Arg Ile Gln Ala Ser Gln Gln Asp Asp Ser Met Arg
    370                 375                 380
Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg
385                 390                 395                 400
Pro Gly Pro Pro Ser Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr
                405                 410                 415
Ser Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Gly Pro Ala
            420                 425                 430
Leu Thr Pro Ile Arg Asp Glu Glu Trp Gly Gly His Ser Pro Arg Ser
        435                 440                 445
Pro Arg Gly Trp Asp Gln Glu Pro Ala Arg Glu Gln Ala Gly Gly Gly
    450                 455                 460
Trp Arg Ala Arg Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp
465                 470                 475                 480
Leu Thr Pro Pro Ser Thr Ala Glu Ser Gly Ser Arg Ser Pro Thr Ser
                485                 490                 495
Asn Gly Gly Arg Ser Arg Ala Tyr Met Pro Pro Arg Ser Arg Ser Arg
            500                 505                 510
Asp Asp Leu Tyr Asp Gln Asp Asp Ser Arg Asp Phe Pro Arg Ser Arg
        515                 520                 525
Asp Pro His Tyr Asp Asp Phe Arg Ser Arg Glu Arg Pro Pro Ala Asp
    530                 535                 540
Pro Arg Ser His His His Arg Thr Arg Asp Pro Arg Asp Asn Gly Ser
545                 550                 555                 560
Arg Ser Gly Asp Leu Pro Tyr Asp Gly Arg Leu Leu Glu Glu Ala Val
                565                 570                 575
Arg Lys Lys Gly Ser Glu Glu Arg Arg Arg Pro His Lys Glu Glu Glu
            580                 585                 590
Glu Glu Ala Tyr Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp
        595                 600                 605
Ser Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser
    610                 615                 620
Arg Glu Ser Leu Val Val
625                 630
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<210> 6
<211> 1954
<212> DNA
<213> Homo sapiens

<220>
<221> allele
<222> 595
<223> 9-3-324 : polymorphic base C or T

<220>
<221> allele
<222> 987
<223> 9-7-325 : polymorphic base A or G

<220>
<221> allele
<222> 1158
<223> 9-9-246 : polymorphic base G or C

<220>
<221> allele
<222> 1454
<223> LSRX9f13-BM : polymorphic base deletion of AGG

<220>
<221> allele
<222> 1875
<223> LSRX9f14-BM : polymorphic base T or G

<400> 6

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tggagtgtgg ctcggaggac cgcggcgggt caagcacctt tctcccccat atctgaaagc      60
atgccctttg tccacgtcgt ttacgctcat taaaacttcc aga atg caa cag gac        115
                                                 Met Gln Gln Asp
                                                 1
gga ctt gga gta ggg aca agg aac gga agt ggg aag ggg agg agc gtg        163
Gly Leu Gly Val Gly Thr Arg Asn Gly Ser Gly Lys Gly Arg Ser Val
5                   10                  15                  20
cac ccc tcc tgg cct tgg tgc gcg ccg cgc ccc cta agg tac ttt gga        211
His Pro Ser Trp Pro Trp Cys Ala Pro Arg Pro Leu Arg Tyr Phe Gly
                25                  30                  35
agg gac gcg cgg gcc aga cgc gcc cag acg gcc gcg atg gcg ctg ttg        259
Arg Asp Ala Arg Ala Arg Arg Ala Gln Thr Ala Ala Met Ala Leu Leu
            40                  45                  50
gcc ggc ggg ctc tcc aga ggg ctg ggc tcc cac ccg gcc gcc gca ggc        307
Ala Gly Gly Leu Ser Arg Gly Leu Gly Ser His Pro Ala Ala Ala Gly
        55                  60                  65
cgg gac gcg gtc gtc ttc gtg tgg ctt ctg ctt agc acc tgg tgc aca        355
Arg Asp Ala Val Val Phe Val Trp Leu Leu Leu Ser Thr Trp Cys Thr
    70                  75                  80
gct cct gcc agg gcc atc cag gtg acc gtg tcc aac ccc tac cac gtg        403
Ala Pro Ala Arg Ala Ile Gln Val Thr Val Ser Asn Pro Tyr His Val
85                  90                  95                  100
gtg atc ctc ttc cag cct gtg acc ctg ccc tgt acc tac cag atg acc        451
Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Thr
                105                 110                 115
tcg acc ccc acg caa ccc atc gtc atc tgg aag tac aag tct ttc tgc        499
Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys
            120                 125                 130
cgg gac cgc atc gcc gat gcc ttc tcc ccg gcc agc gtc gac aac cag        547
Arg Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln
        135                 140                 145
ctc aat gcc cag ctg gca gcc ggg aac cca ggc tac aac ccc tac gty        595
Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val
    150                 155                 160
gag tgc cag gac agc gtg cgc acc gtc agg gtc gtg gcc acc aag cag        643
Glu Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln
165                 170                 175                 180
```

```
ggc aac gct gtg acc ctg gga gat tac tac cag ggc cgg agg att acc      691
Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr
                185                 190                 195
atc acc gga aat gct gac ctg acc ttt gac cag acg gcg tgg ggg gac      739
Ile Thr Gly Asn Ala Asp Leu Thr Phe Asp Gln Thr Ala Trp Gly Asp
            200                 205                 210
agt ggt gtg tat tac tgc tcc gtg gtc tca gcc cag gac ctc cag ggg      787
Ser Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Gln Gly
        215                 220                 225
aac aat gag gcc tac gca gag ctc atc gtc ctt gtg tat gcc gcc ggc      835
Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Val Tyr Ala Ala Gly
    230                 235                 240
aaa gca gcc acc tca ggt gtt ccc agc att tat gcc ccc agc acc tat      883
Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Thr Tyr
245                 250                 255                 260
gcc cac ctg tct ccc gcc aag acc cca ccc cca cca gct atg att ccc      931
Ala His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Ala Met Ile Pro
                265                 270                 275
atg ggc cct gcc tac aac ggg tac cct gga gga tac cct gga gac gtt      979
Met Gly Pro Ala Tyr Asn Gly Tyr Pro Gly Gly Tyr Pro Gly Asp Val
            280                 285                 290
gac agg art agc tca gct ggt ggc caa ggc tcc tat gta ccc ctg ctt     1027
Asp Arg Xaa Ser Ser Ala Gly Gly Gln Gly Ser Tyr Val Pro Leu Leu
        295                 300                 305
cgg gac acg gac agc agt gtg gcc tct gaa gtc cgc agt ggc tac agg     1075
Arg Asp Thr Asp Ser Ser Val Ala Ser Glu Val Arg Ser Gly Tyr Arg
    310                 315                 320
att cag gcc agc cag cag gac gac tcc atg cgg gtc ctg tac tac atg     1123
Ile Gln Ala Ser Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met
325                 330                 335                 340
gag aag gag ctg gcc aac ttc gac cct tct cga cst ggc ccc ccc agt     1171
Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Xaa Gly Pro Pro Ser
                345                 350                 355
ggc cgt gtg gag cgg gcc atg agt gaa gtc acc tcc ctc cac gag gac     1219
Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu His Glu Asp
            360                 365                 370
gac tgg cga tct cgg cct tcc cgg ggc cct gcc ctc acc ccg atc cgg     1267
Asp Trp Arg Ser Arg Pro Ser Arg Gly Pro Ala Leu Thr Pro Ile Arg
        375                 380                 385
gat gag gag tgg ggt ggc cac tcc ccc cgg agt ccc agg gga tgg gac     1315
Asp Glu Glu Trp Gly Gly His Ser Pro Arg Ser Pro Arg Gly Trp Asp
    390                 395                 400
cag gag ccc gcc agg gag cag gca ggc ggg ggc tgg cgg gcc agg cgg     1363
Gln Glu Pro Ala Arg Glu Gln Ala Gly Gly Gly Trp Arg Ala Arg Arg
405                 410                 415                 420
ccc cgg gcc cgc tcc gtg gac gcc ctg gac gac ctc acc ccg ccg agc     1411
Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Leu Thr Pro Pro Ser
                425                 430                 435
acc gcc gag tca ggg agc agg tct ccc acg agt aat ggt ggg aga agc     1459
Thr Ala Glu Ser Gly Ser Arg Ser Pro Thr Ser Asn Gly Gly Arg Ser
            440                 445                 450
cgg gcc tac atg ccc ccg cgg agc cgc agc cgg gac gac ctc tat gac     1507
Arg Ala Tyr Met Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp
        455                 460                 465
caa gac gac tcg agg gac ttc cca cgc tcc cgg gac ccc cac tac gac     1555
Gln Asp Asp Ser Arg Asp Phe Pro Arg Ser Arg Asp Pro His Tyr Asp
    470                 475                 480
gac ttc agg tct cgg gag cgc cct cct gcc gac ccc agg tcc cac cac     1603
Asp Phe Arg Ser Arg Glu Arg Pro Pro Ala Asp Pro Arg Ser His His
485                 490                 495                 500
cac cgt acc cgg gac cct cgg gac aac ggc tcc agg tcc ggg gac ctc     1651
His Arg Thr Arg Asp Pro Arg Asp Asn Gly Ser Arg Ser Gly Asp Leu
                505                 510                 515
```

```
ccc tat gat ggg cgg cta ctg gag gag gct gtg agg aag aag ggg tcg      1699
Pro Tyr Asp Gly Arg Leu Leu Glu Glu Ala Val Arg Lys Lys Gly Ser
            520                 525                 530
gag gag agg agg aga ccc cac aag gag gag gag gaa gag gcc tac tac      1747
Glu Glu Arg Arg Arg Pro His Lys Glu Glu Glu Glu Glu Ala Tyr Tyr
        535                 540                 545
ccg ccc gcg ccg ccc ccg tac tcg gag acc gac tcg cag gcg tcc cga      1795
Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg
    550                 555                 560
gag cgc agg ctc aag aag aac ttg gcc ctg agt cgg gaa agt tta gtc      1843
Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val
565                 570                 575                 580
gtc tga tctgacgttt tctacgtagc ttttgkattt ttttttttaa tttgaaggaa      1899
Val  *
cactgatgaa gccctgccat acccctcccg agtctaataa aacgtataat cacaa       1954

<210> 7
<211> 581
<212> PRT
<213> Homo sapiens

<220>
<221> VARIANT
<222> 295
<223> 9-7-325  :  polymorphic  amino  acid  Ser  or  Asn

<220>
<221> VARIANT
<222> 352
<223> 9-9-246  :  polymorphic  amino  acid  Pro  or  Arg

<220>
<221> VARIANT
<222> 451
<223> LSRX9f13-BM  :  polymorphic  amino  acid  deletion  of  Arg

<400> 7
Met Gln Gln Asp Gly Leu Gly Val Gly Thr Arg Asn Gly Ser Gly Lys
1               5                   10                  15
Gly Arg Ser Val His Pro Ser Trp Pro Trp Cys Ala Pro Arg Pro Leu
            20                  25                  30
Arg Tyr Phe Gly Arg Asp Ala Arg Ala Arg Arg Ala Gln Thr Ala Ala
        35                  40                  45
Met Ala Leu Leu Ala Gly Gly Leu Ser Arg Gly Leu Gly Ser His Pro
    50                  55                  60
Ala Ala Ala Gly Arg Asp Ala Val Val Phe Val Trp Leu Leu Leu Ser
65                  70                  75                  80
Thr Trp Cys Thr Ala Pro Ala Arg Ala Ile Gln Val Thr Val Ser Asn
                85                  90                  95
Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr
            100                 105                 110
Tyr Gln Met Thr Ser Thr Pro Thr Gln Pro Ile Val Ile Trp Lys Tyr
        115                 120                 125
Lys Ser Phe Cys Arg Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser
    130                 135                 140
Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr
145                 150                 155                 160
Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg Thr Val Arg Val Val
                165                 170                 175
Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly
            180                 185                 190
Arg Arg Ile Thr Ile Thr Gly Asn Ala Asp Leu Thr Phe Asp Gln Thr
        195                 200                 205
```

```
Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln
    210                 215                 220
Asp Leu Gln Gly Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Val
225                 230                 235                 240
Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala
                245                 250                 255
Pro Ser Thr Tyr Ala His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro
            260                 265                 270
Ala Met Ile Pro Met Gly Pro Ala Tyr Asn Gly Tyr Pro Gly Gly Tyr
        275                 280                 285
Pro Gly Asp Val Asp Arg Ser Ser Ser Ala Gly Gly Gln Gly Ser Tyr
    290                 295                 300
Val Pro Leu Leu Arg Asp Thr Asp Ser Ser Val Ala Ser Glu Val Arg
305                 310                 315                 320
Ser Gly Tyr Arg Ile Gln Ala Ser Gln Gln Asp Asp Ser Met Arg Val
                325                 330                 335
Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Pro
            340                 345                 350
Gly Pro Pro Ser Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser
        355                 360                 365
Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Gly Pro Ala Leu
    370                 375                 380
Thr Pro Ile Arg Asp Glu Glu Trp Gly Gly His Ser Pro Arg Ser Pro
385                 390                 395                 400
Arg Gly Trp Asp Gln Glu Pro Ala Arg Glu Gln Ala Gly Gly Gly Trp
                405                 410                 415
Arg Ala Arg Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Leu
            420                 425                 430
Thr Pro Pro Ser Thr Ala Glu Ser Gly Ser Arg Ser Pro Thr Ser Asn
        435                 440                 445
Gly Gly Arg Ser Arg Ala Tyr Met Pro Pro Arg Ser Arg Ser Arg Asp
    450                 455                 460
Asp Leu Tyr Asp Gln Asp Asp Ser Arg Asp Phe Pro Arg Ser Arg Asp
465                 470                 475                 480
Pro His Tyr Asp Asp Phe Arg Ser Arg Glu Arg Pro Pro Ala Asp Pro
                485                 490                 495
Arg Ser His His His Arg Thr Arg Asp Pro Arg Asp Asn Gly Ser Arg
            500                 505                 510
Ser Gly Asp Leu Pro Tyr Asp Gly Arg Leu Leu Glu Glu Ala Val Arg
        515                 520                 525
Lys Lys Gly Ser Glu Glu Arg Arg Arg Pro His Lys Glu Glu Glu Glu
    530                 535                 540
Glu Ala Tyr Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser
545                 550                 555                 560
Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser Arg
                565                 570                 575
Glu Ser Leu Val Val
            580
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<210> 8
<211> 2097
<212> DNA
<213> Rattus norvegicus

<400> 8

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accgctcacc aggtcagttg tccccggaaa gccgaaggca tgagcttcgc ccaagttctt      60
tttatgggtt agaactcctc cagagcgggg gaaaaaggac ttggaatagg ggcgggacgg     120
agcacgcacc cttctccgcc ttggttctcg ccgcgccccc tactctcggg atacttggga     180
ggggacgcgc gggcaccgtc gctgctagac ggccgcg atg gcg ccg gcg gcc ggc       235
                                          Met Ala Pro Ala Ala Gly
                                          1               5
gcg tgt gct ggg gcg cct gac tcc cac cca gct acc gtg gtc ttc gtg       283
Ala Cys Ala Gly Ala Pro Asp Ser His Pro Ala Thr Val Val Phe Val
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          10                  15                  20
tgt ctc ttt ctc atc att ttc tgc cca gac cct gcc agt gcc atc cag      331
Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp Pro Ala Ser Ala Ile Gln
        25                  30                  35
gtg act gtg tct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg      379
Val Thr Val Ser Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val
    40                  45                  50
acc ctg ccc tgc acc tat cag atg agc aac act ctc aca gtc ccc atc      427
Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Val Pro Ile
55                  60                  65                  70
gtg atc tgg aag tac aag tca ttc tgc cgg gac cgt att gcc gat gcc      475
Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg Asp Arg Ile Ala Asp Ala
                75                  80                  85
ttc tct cct gcc agt gtg gac aac cag cta aat gcc cag ttg gca gct      523
Phe Ser Pro Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala
            90                  95                  100
ggc aac ccc ggc tac aac ccc tat gtg gag tgc cag gac agt gta cgc      571
Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg
        105                 110                 115
act gtc agg gtg gtg gcc acc aaa cag ggc aat gcg gtg acc ctg gga      619
Thr Val Arg Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly
    120                 125                 130
gac tac tac caa ggc agg agg atc acc ata aca gga aat gct gac ctg      667
Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Asp Leu
135                 140                 145                 150
acc ttc gag cag aca gcc tgg gga gac agt gga gtg tat tac tgc tct      715
Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser
                155                 160                 165
gtg gtc tcg gcc caa gat ctg gat gga aac aac gag gcg tac gca gag      763
Val Val Ser Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu
            170                 175                 180
ctc atc gtc ctt ggc agg acc tca gag gcc cct gag ctc cta cct ggt      811
Leu Ile Val Leu Gly Arg Thr Ser Glu Ala Pro Glu Leu Leu Pro Gly
        185                 190                 195
ttt cgg gcg ggg ccc ttg gaa gat tgg ctc ttt gtg gtc gtg gtc tgc      859
Phe Arg Ala Gly Pro Leu Glu Asp Trp Leu Phe Val Val Val Val Cys
    200                 205                 210
ctg gcg agc ctc ctc ctc ttc ctc ctc ctg ggc atc tgc tgg tgc cag      907
Leu Ala Ser Leu Leu Leu Phe Leu Leu Leu Gly Ile Cys Trp Cys Gln
215                 220                 225                 230
tgc tgt cct cac acc tgc tgc tgc tat gtc cga tgt ccc tgc tgc cca      955
Cys Cys Pro His Thr Cys Cys Cys Tyr Val Arg Cys Pro Cys Cys Pro
                235                 240                 245
gac aag tgc tgt tgc cct gag gct ctt tat gct gct ggc aaa gca gcc     1003
Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr Ala Ala Gly Lys Ala Ala
            250                 255                 260
acc tca ggt gtc ccg agc atc tat gcc ccc agc atc tat acc cac ctc     1051
Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr Thr His Leu
        265                 270                 275
tca cct gcc aag acc cca cca cct ccg cct gcc atg att ccc atg ggc     1099
Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro Ala Met Ile Pro Met Gly
    280                 285                 290
cct ccc tat ggg tac cct gga gac ttt gac aga cat agc tca gtt ggt     1147
Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp Arg His Ser Ser Val Gly
295                 300                 305                 310
ggc cac agc tcc caa gta ccc ctg ctg cgt gac gtg gat ggc agt gta     1195
Gly His Ser Ser Gln Val Pro Leu Leu Arg Asp Val Asp Gly Ser Val
                315                 320                 325
tct tca gaa gta cga agt ggc tac agg atc cag gct aac cag caa gat     1243
Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn Gln Gln Asp
            330                 335                 340
gac tcc atg agg gtc cta tac tat atg gag aaa gag cta gcc aac ttt     1291
Asp Ser Met Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe
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        345                 350                 355
gac cct tcc cga cct ggc cct ccc aat ggc aga gtg gaa cgg gcc atg     1339
Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly Arg Val Glu Arg Ala Met
    360                 365                 370
agt gaa gta acc tcc ctc cat gaa gat gac tgg cga tcg agg cct tcc     1387
Ser Glu Val Thr Ser Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser
375                 380                 385                 390
agg gct cct gcc ctc acc ccc atc agg gat gag gag tgg aat cgc cac     1435
Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp Asn Arg His
                395                 400                 405
tcc cca cag agt ccc aga aca tgg gag cag gaa ccc ctt caa gaa caa     1483
Ser Pro Gln Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu Gln Glu Gln
            410                 415                 420
cca agg ggt ggt tgg ggg tct gga cgc cct cgg gcc cgc tct gtg gat     1531
Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg Ser Val Asp
        425                 430                 435
gct cta gat gat atc aac cgg cct ggc tcc act gaa tca gga cgg tct     1579
Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser Gly Arg Ser
    440                 445                 450
tct ccc cca agt agt gga cgg aga gga cgg gcc tat gca cct cca aga     1627
Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala Pro Pro Arg
455                 460                 465                 470
agt cgc agc cgg gat gac ctc tat gac ccg gac gat cct agg gac ttg     1675
Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro Arg Asp Leu
                475                 480                 485
cca cat tcc cga gat ccc cac tat tat gac gac atc agg tct aga gat     1723
Pro His Ser Arg Asp Pro His Tyr Tyr Asp Asp Ile Arg Ser Arg Asp
            490                 495                 500
cca cgt gct gac ccc aga tcc cgt cag cga tcc cga gat cct cgg gat     1771
Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg Ser Arg Asp Pro Arg Asp
        505                 510                 515
gct ggc ttc agg tca agg gac cct cag tat gat ggg cga cta tta gaa     1819
Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg Leu Leu Glu
    520                 525                 530
gag gct tta aag aaa aag ggg tcg ggc gag aga agg agg gtt tac agg     1867
Glu Ala Leu Lys Lys Lys Gly Ser Gly Glu Arg Arg Arg Val Tyr Arg
535                 540                 545                 550
gag gaa gaa gag gaa gag gag ggc caa tac ccc cca gca cct cca cct     1915
Glu Glu Glu Glu Glu Glu Glu Gly Gln Tyr Pro Pro Ala Pro Pro Pro
                555                 560                 565
tac tca gag act gac tcg cag gcc tca cgg gag agg agg ctg aaa aag     1963
Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys
            570                 575                 580
aat ttg gcc ctg agt cgg gaa agt tta gtc gtc tga tccacgtttt          2009
Asn Leu Ala Leu Ser Arg Glu Ser Leu Val Val  *
        585                 590
gtatgtagct tttgtacttt ttttttaatt ggaatcaata ttgatgaaac ttcaagccta   2069
ataaaatgtc taatcacaaa aaaaaaaa                                      2097

<210> 9
<211> 593
<212> PRT
<213> Rattus norvegicus

<400> 9
Met Ala Pro Ala Ala Gly Ala Cys Ala Gly Ala Pro Asp Ser His Pro
1               5                   10                  15
Ala Thr Val Val Phe Val Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp
            20                  25                  30
Pro Ala Ser Ala Ile Gln Val Thr Val Ser Asp Pro Tyr His Val Val
        35                  40                  45
Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn
    50                  55                  60
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Thr Leu Thr Val Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65                  70                  75                  80
Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
                85                  90                  95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
            100                 105                 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
        115                 120                 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
    130                 135                 140
Thr Gly Asn Ala Asp Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145                 150                 155                 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
                165                 170                 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Gly Arg Thr Ser Glu Ala
            180                 185                 190
Pro Glu Leu Leu Pro Gly Phe Arg Ala Gly Pro Leu Glu Asp Trp Leu
        195                 200                 205
Phe Val Val Val Val Cys Leu Ala Ser Leu Leu Leu Phe Leu Leu Leu
    210                 215                 220
Gly Ile Cys Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr Val
225                 230                 235                 240
Arg Cys Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr
                245                 250                 255
Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro
            260                 265                 270
Ser Ile Tyr Thr His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro
        275                 280                 285
Ala Met Ile Pro Met Gly Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp
    290                 295                 300
Arg His Ser Ser Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg
305                 310                 315                 320
Asp Val Asp Gly Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile
                325                 330                 335
Gln Ala Asn Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu
            340                 345                 350
Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly
        355                 360                 365
Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp
    370                 375                 380
Trp Arg Ser Arg Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp
385                 390                 395                 400
Glu Glu Trp Asn Arg His Ser Pro Gln Ser Pro Arg Thr Trp Glu Gln
                405                 410                 415
Glu Pro Leu Gln Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro
            420                 425                 430
Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser
        435                 440                 445
Thr Glu Ser Gly Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg
    450                 455                 460
Ala Tyr Ala Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro
465                 470                 475                 480
Asp Asp Pro Arg Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp
                485                 490                 495
Asp Ile Arg Ser Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg
            500                 505                 510
Ser Arg Asp Pro Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr
        515                 520                 525
Asp Gly Arg Leu Leu Glu Glu Ala Leu Lys Lys Lys Gly Ser Gly Glu
    530                 535                 540
Arg Arg Arg Val Tyr Arg Glu Glu Glu Glu Glu Glu Glu Gly Gln Tyr
545                 550                 555                 560
Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg
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                565                 570                 575
Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val
            580                 585                 590
Val

<210> 10
<211> 2040
<212> DNA
<213> Rattus norvegicus

<400> 10
accgctcacc aggtcagttg tccccggaaa gccgaaggca tgagcttcgc ccaagttctt      60
tttatgggtt agaactcctc cagagcgggg gaaaaaggac ttggaatagg ggcgggacgg     120
agcacgcacc cttctccgcc ttggttctcg ccgcgccccc tactctcggg atacttggga     180
ggggacgcgc gggcaccgtc gctgctagac ggccgcg atg gcg ccg gcg gcc ggc       235
                                          Met Ala Pro Ala Ala Gly
                                          1               5
gcg tgt gct ggg gcg cct gac tcc cac cca gct acc gtg gtc ttc gtg       283
Ala Cys Ala Gly Ala Pro Asp Ser His Pro Ala Thr Val Val Phe Val
            10                  15                  20
tgt ctc ttt ctc atc att ttc tgc cca gac cct gcc agt gcc atc cag       331
Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp Pro Ala Ser Ala Ile Gln
        25                  30                  35
gtg act gtg tct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg       379
Val Thr Val Ser Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val
    40                  45                  50
acc ctg ccc tgc acc tat cag atg agc aac act ctc aca gtc ccc atc       427
Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Val Pro Ile
55                  60                  65                  70
gtg atc tgg aag tac aag tca ttc tgc cgg gac cgt att gcc gat gcc       475
Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg Asp Arg Ile Ala Asp Ala
                75                  80                  85
ttc tct cct gcc agt gtg gac aac cag cta aat gcc cag ttg gca gct       523
Phe Ser Pro Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala
            90                  95                  100
ggc aac ccc ggc tac aac ccc tat gtg gag tgc cag gac agt gta cgc       571
Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg
        105                 110                 115
act gtc agg gtg gtg gcc acc aaa cag ggc aat gcg gtg acc ctg gga       619
Thr Val Arg Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly
    120                 125                 130
gac tac tac caa ggc agg agg atc acc ata aca gga aat gct gac ctg       667
Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Asp Leu
135                 140                 145                 150
acc ttc gag cag aca gcc tgg gga gac agt gga gtg tat tac tgc tct       715
Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser
                155                 160                 165
gtg gtc tcg gcc caa gat ctg gat gga aac aac gag gcg tac gca gag       763
Val Val Ser Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu
            170                 175                 180
ctc atc gtc ctt gat tgg ctc ttt gtg gtc gtg gtc tgc ctg gcg agc       811
Leu Ile Val Leu Asp Trp Leu Phe Val Val Val Val Cys Leu Ala Ser
        185                 190                 195
ctc ctc ctc ttc ctc ctc ctg ggc atc tgc tgg tgc cag tgc tgt cct       859
Leu Leu Leu Phe Leu Leu Leu Gly Ile Cys Trp Cys Gln Cys Cys Pro
    200                 205                 210
cac acc tgc tgc tgc tat gtc cga tgt ccc tgc tgc cca gac aag tgc       907
His Thr Cys Cys Cys Tyr Val Arg Cys Pro Cys Cys Pro Asp Lys Cys
215                 220                 225                 230
tgt tgc cct gag gct ctt tat gct gct ggc aaa gca gcc acc tca ggt       955
Cys Cys Pro Glu Ala Leu Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly
                235                 240                 245
gtc ccg agc atc tat gcc ccc agc atc tat acc cac ctc tca cct gcc      1003
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Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr Thr His Leu Ser Pro Ala
            250                 255                 260
aag acc cca cca cct ccg cct gcc atg att ccc atg ggc cct ccc tat     1051
Lys Thr Pro Pro Pro Pro Pro Ala Met Ile Pro Met Gly Pro Pro Tyr
        265                 270                 275
ggg tac cct gga gac ttt gac aga cat agc tca gtt ggt ggc cac agc     1099
Gly Tyr Pro Gly Asp Phe Asp Arg His Ser Ser Val Gly Gly His Ser
    280                 285                 290
tcc caa gta ccc ctg ctg cgt gac gtg gat ggc agt gta tct tca gaa     1147
Ser Gln Val Pro Leu Leu Arg Asp Val Asp Gly Ser Val Ser Ser Glu
295                 300                 305                 310
gta cga agt ggc tac agg atc cag gct aac cag caa gat gac tcc atg     1195
Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn Gln Gln Asp Asp Ser Met
                315                 320                 325
agg gtc cta tac tat atg gag aaa gag cta gcc aac ttt gac cct tcc     1243
Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser
            330                 335                 340
cga cct ggc cct ccc aat ggc aga gtg gaa cgg gcc atg agt gaa gta     1291
Arg Pro Gly Pro Pro Asn Gly Arg Val Glu Arg Ala Met Ser Glu Val
        345                 350                 355
acc tcc ctc cat gaa gat gac tgg cga tcg agg cct tcc agg gct cct     1339
Thr Ser Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Ala Pro
    360                 365                 370
gcc ctc acc ccc atc agg gat gag gag tgg aat cgc cac tcc cca cag     1387
Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp Asn Arg His Ser Pro Gln
375                 380                 385                 390
agt ccc aga aca tgg gag cag gaa ccc ctt caa gaa caa cca agg ggt     1435
Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu Gln Glu Gln Pro Arg Gly
                395                 400                 405
ggt tgg ggg tct gga cgc cct cgg gcc cgc tct gtg gat gct cta gat     1483
Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp
            410                 415                 420
gat atc aac cgg cct ggc tcc act gaa tca gga cgg tct tct ccc cca     1531
Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser Gly Arg Ser Ser Pro Pro
        425                 430                 435
agt agt gga cgg aga gga cgg gcc tat gca cct cca aga agt cgc agc     1579
Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala Pro Pro Arg Ser Arg Ser
    440                 445                 450
cgg gat gac ctc tat gac ccg gac gat cct agg gac ttg cca cat tcc     1627
Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro Arg Asp Leu Pro His Ser
455                 460                 465                 470
cga gat ccc cac tat tat gac gac atc agg tct aga gat cca cgt gct     1675
Arg Asp Pro His Tyr Tyr Asp Asp Ile Arg Ser Arg Asp Pro Arg Ala
                475                 480                 485
gac ccc aga tcc cgt cag cga tcc cga gat cct cgg gat gct ggc ttc     1723
Asp Pro Arg Ser Arg Gln Arg Ser Arg Asp Pro Arg Asp Ala Gly Phe
            490                 495                 500
agg tca agg gac cct cag tat gat ggg cga cta tta gaa gag gct tta     1771
Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg Leu Leu Glu Glu Ala Leu
        505                 510                 515
aag aaa aag ggg tcg ggc gag aga agg agg gtt tac agg gag gaa gaa     1819
Lys Lys Lys Gly Ser Gly Glu Arg Arg Arg Val Tyr Arg Glu Glu Glu
    520                 525                 530
gag gaa gag gag ggc caa tac ccc cca gca cct cca cct tac tca gag     1867
Glu Glu Glu Glu Gly Gln Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu
535                 540                 545                 550
act gac tcg cag gcc tca cgg gag agg agg ctg aaa aag aat ttg gcc     1915
Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala
                555                 560                 565
ctg agt cgg gaa agt tta gtc gtc tga tccacgtttt gtatgtagct           1962
Leu Ser Arg Glu Ser Leu Val Val  *
            570                 575
tttgtacttt ttttttaatt ggaatcaata ttgatgaaac ttcaagccta ataaaatgtc   2022
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taatcacaaa aaaaaaaa 2040

<210> 11
<211> 574
<212> PRT
<213> Rattus norvegicus

<400> 11
Met Ala Pro Ala Ala Gly Ala Cys Ala Gly Ala Pro Asp Ser His Pro
1 5 10 15
Ala Thr Val Val Phe Val Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp
20 25 30
Pro Ala Ser Ala Ile Gln Val Thr Val Ser Asp Pro Tyr His Val Val
35 40 45
Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn
50 55 60
Thr Leu Thr Val Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65 70 75 80
Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
85 90 95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
100 105 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
115 120 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
130 135 140
Thr Gly Asn Ala Asp Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145 150 155 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
165 170 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Asp Trp Leu Phe Val Val
180 185 190
Val Val Cys Leu Ala Ser Leu Leu Leu Phe Leu Leu Leu Gly Ile Cys
195 200 205
Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr Val Arg Cys Pro
210 215 220
Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr Ala Ala Gly
225 230 235 240
Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr
245 250 255
Thr His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro Ala Met Ile
260 265 270
Pro Met Gly Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp Arg His Ser
275 280 285
Ser Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg Asp Val Asp
290 295 300
Gly Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn
305 310 315 320
Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu
325 330 335
Ala Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly Arg Val Glu
340 345 350
Arg Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp Trp Arg Ser
355 360 365
Arg Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp
370 375 380
Asn Arg His Ser Pro Gln Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu
385 390 395 400
Gln Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg
405 410 415
Ser Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser
420 425 430
Gly Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala

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        435                 440                 445
Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro
    450                 455                 460
Arg Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp Asp Ile Arg
465                 470                 475                 480
Ser Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg Ser Arg Asp
                485                 490                 495
Pro Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg
            500                 505                 510
Leu Leu Glu Glu Ala Leu Lys Lys Lys Gly Ser Gly Glu Arg Arg Arg
        515                 520                 525
Val Tyr Arg Glu Glu Glu Glu Glu Glu Glu Gly Gln Tyr Pro Pro Ala
    530                 535                 540
Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg
545                 550                 555                 560
Leu Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val Val
                565                 570
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<210> 12
<211> 1893
<212> DNA
<213> Rattus norvegicus

<400> 12

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agcacgcacc cttctccgcc ttggttctcg ccgcgccccc tactctcggg atacttggga     180
ggggacgcgc gggcaccgtc gctgctagac ggccgcg atg gcg ccg gcg gcc ggc       235
                                          Met Ala Pro Ala Ala Gly
                                          1               5
gcg tgt gct ggg gcg cct gac tcc cac cca gct acc gtg gtc ttc gtg       283
Ala Cys Ala Gly Ala Pro Asp Ser His Pro Ala Thr Val Val Phe Val
            10                  15                  20
tgt ctc ttt ctc atc att ttc tgc cca gac cct gcc agt gcc atc cag       331
Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp Pro Ala Ser Ala Ile Gln
        25                  30                  35
gtg act gtg tct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg       379
Val Thr Val Ser Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val
    40                  45                  50
acc ctg ccc tgc acc tat cag atg agc aac act ctc aca gtc ccc atc       427
Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Val Pro Ile
55                  60                  65                  70
gtg atc tgg aag tac aag tca ttc tgc cgg gac cgt att gcc gat gcc       475
Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg Asp Arg Ile Ala Asp Ala
                75                  80                  85
ttc tct cct gcc agt gtg gac aac cag cta aat gcc cag ttg gca gct       523
Phe Ser Pro Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala
            90                  95                  100
ggc aac ccc ggc tac aac ccc tat gtg gag tgc cag gac agt gta cgc       571
Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg
        105                 110                 115
act gtc agg gtg gtg gcc acc aaa cag ggc aat gcg gtg acc ctg gga       619
Thr Val Arg Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly
    120                 125                 130
gac tac tac caa ggc agg agg atc acc ata aca gga aat gct gac ctg       667
Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Asp Leu
135                 140                 145                 150
acc ttc gag cag aca gcc tgg gga gac agt gga gtg tat tac tgc tct       715
Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser
                155                 160                 165
gtg gtc tcg gcc caa gat ctg gat gga aac aac gag gcg tac gca gag       763
Val Val Ser Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu
            170                 175                 180
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ctc atc gtc ctt gtt tat gct gct ggc aaa gca gcc acc tca ggt gtc      811
Leu Ile Val Leu Val Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val
        185                 190                 195
ccg agc atc tat gcc ccc agc atc tat acc cac ctc tca cct gcc aag      859
Pro Ser Ile Tyr Ala Pro Ser Ile Tyr Thr His Leu Ser Pro Ala Lys
    200                 205                 210
acc cca cca cct ccg cct gcc atg att ccc atg ggc cct ccc tat ggg      907
Thr Pro Pro Pro Pro Pro Ala Met Ile Pro Met Gly Pro Pro Tyr Gly
215                 220                 225                 230
tac cct gga gac ttt gac aga cat agc tca gtt ggt ggc cac agc tcc      955
Tyr Pro Gly Asp Phe Asp Arg His Ser Ser Val Gly Gly His Ser Ser
                235                 240                 245
caa gta ccc ctg ctg cgt gac gtg gat ggc agt gta tct tca gaa gta     1003
Gln Val Pro Leu Leu Arg Asp Val Asp Gly Ser Val Ser Ser Glu Val
            250                 255                 260
cga agt ggc tac agg atc cag gct aac cag caa gat gac tcc atg agg     1051
Arg Ser Gly Tyr Arg Ile Gln Ala Asn Gln Gln Asp Asp Ser Met Arg
        265                 270                 275
gtc cta tac tat atg gag aaa gag cta gcc aac ttt gac cct tcc cga     1099
Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg
    280                 285                 290
cct ggc cct ccc aat ggc aga gtg gaa cgg gcc atg agt gaa gta acc     1147
Pro Gly Pro Pro Asn Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr
295                 300                 305                 310
tcc ctc cat gaa gat gac tgg cga tcg agg cct tcc agg gct cct gcc     1195
Ser Leu His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Ala Pro Ala
                315                 320                 325
ctc acc ccc atc agg gat gag gag tgg aat cgc cac tcc cca cag agt     1243
Leu Thr Pro Ile Arg Asp Glu Glu Trp Asn Arg His Ser Pro Gln Ser
            330                 335                 340
ccc aga aca tgg gag cag gaa ccc ctt caa gaa caa cca agg ggt ggt     1291
Pro Arg Thr Trp Glu Gln Glu Pro Leu Gln Glu Gln Pro Arg Gly Gly
        345                 350                 355
tgg ggg tct gga cgc cct cgg gcc cgc tct gtg gat gct cta gat gat     1339
Trp Gly Ser Gly Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp
    360                 365                 370
atc aac cgg cct ggc tcc act gaa tca gga cgg tct tct ccc cca agt     1387
Ile Asn Arg Pro Gly Ser Thr Glu Ser Gly Arg Ser Ser Pro Pro Ser
375                 380                 385                 390
agt gga cgg aga gga cgg gcc tat gca cct cca aga agt cgc agc cgg     1435
Ser Gly Arg Arg Gly Arg Ala Tyr Ala Pro Pro Arg Ser Arg Ser Arg
                395                 400                 405
gat gac ctc tat gac ccg gac gat cct agg gac ttg cca cat tcc cga     1483
Asp Asp Leu Tyr Asp Pro Asp Asp Pro Arg Asp Leu Pro His Ser Arg
            410                 415                 420
gat ccc cac tat tat gac gac atc agg tct aga gat cca cgt gct gac     1531
Asp Pro His Tyr Tyr Asp Asp Ile Arg Ser Arg Asp Pro Arg Ala Asp
        425                 430                 435
ccc aga tcc cgt cag cga tcc cga gat cct cgg gat gct ggc ttc agg     1579
Pro Arg Ser Arg Gln Arg Ser Arg Asp Pro Arg Asp Ala Gly Phe Arg
    440                 445                 450
tca agg gac cct cag tat gat ggg cga cta tta gaa gag gct tta aag     1627
Ser Arg Asp Pro Gln Tyr Asp Gly Arg Leu Leu Glu Glu Ala Leu Lys
455                 460                 465                 470
aaa aag ggg tcg ggc gag aga agg agg gtt tac agg gag gaa gaa gag     1675
Lys Lys Gly Ser Gly Glu Arg Arg Arg Val Tyr Arg Glu Glu Glu Glu
                475                 480                 485
gaa gag gag ggc caa tac ccc cca gca cct cca cct tac tca gag act     1723
Glu Glu Glu Gly Gln Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr
            490                 495                 500
gac tcg cag gcc tca cgg gag agg agg ctg aaa aag aat ttg gcc ctg     1771
Asp Ser Gln Ala Ser Arg Glu Arg Arg Leu Lys Lys Asn Leu Ala Leu
        505                 510                 515
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agt cgg gaa agt tta gtc gtc tga tccacgtttt gtatgtagct tttgtacttt    1825
Ser Arg Glu Ser Leu Val Val  *
    520                 525
ttttttaatt ggaatcaata ttgatgaaac ttcaagccta ataaaatgtc taatcacaaa   1885
aaaaaaaa                                                            1893

<210> 13
<211> 525
<212> PRT
<213> Rattus norvegicus

<400> 13
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1               5                  10                  15
Ala Thr Val Val Phe Val Cys Leu Phe Leu Ile Ile Phe Cys Pro Asp
            20                  25                  30
Pro Ala Ser Ala Ile Gln Val Thr Val Ser Asp Pro Tyr His Val Val
        35                  40                  45
Ile Leu Phe Gln Pro Val Thr Leu Pro Cys Thr Tyr Gln Met Ser Asn
    50                  55                  60
Thr Leu Thr Val Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65                  70                  75                  80
Asp Arg Ile Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
                85                  90                  95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
            100                 105                 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
        115                 120                 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
    130                 135                 140
Thr Gly Asn Ala Asp Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145                 150                 155                 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
                165                 170                 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Val Tyr Ala Ala Gly Lys
            180                 185                 190
Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr Thr
        195                 200                 205
His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro Ala Met Ile Pro
    210                 215                 220
Met Gly Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp Arg His Ser Ser
225                 230                 235                 240
Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg Asp Val Asp Gly
                245                 250                 255
Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn Gln
            260                 265                 270
Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala
        275                 280                 285
Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly Arg Val Glu Arg
    290                 295                 300
Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp Trp Arg Ser Arg
305                 310                 315                 320
Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp Asn
                325                 330                 335
Arg His Ser Pro Gln Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu Gln
            340                 345                 350
Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg Ser
        355                 360                 365
Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser Gly
    370                 375                 380
Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala Pro
385                 390                 395                 400
Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro Arg
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                405                 410                 415
Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp Asp Ile Arg Ser
            420                 425                 430
Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg Ser Arg Asp Pro
        435                 440                 445
Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg Leu
    450                 455                 460
Leu Glu Glu Ala Leu Lys Lys Lys Gly Ser Gly Glu Arg Arg Arg Val
465                 470                 475                 480
Tyr Arg Glu Glu Glu Glu Glu Glu Glu Gly Gln Tyr Pro Pro Ala Pro
                485                 490                 495
Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg Leu
            500                 505                 510
Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val Val
        515                 520                 525
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<210> 14
<211> 1886
<212> DNA
<213> Mus musculus

<400> 14

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gcaccgtcgc tgctagacgg ccgcg atg gcg ccg gcg gcc agc gcg tgt gct      52
                              Met Ala Pro Ala Ala Ser Ala Cys Ala
                              1               5
ggg gcg cct ggc tcc cac ccg gcc acc acg atc ttc gtg tgt ctt ttt     100
Gly Ala Pro Gly Ser His Pro Ala Thr Thr Ile Phe Val Cys Leu Phe
10                  15                  20                  25
ctc atc att tac tgc cca gac cgt gcc agt gcc atc cag gtg acc gtg     148
Leu Ile Ile Tyr Cys Pro Asp Arg Ala Ser Ala Ile Gln Val Thr Val
                30                  35                  40
cct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg aca cta cac     196
Pro Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu His
            45                  50                  55
tgc acc tac cag atg agc aat acc ctc aca gcc cct atc gtg atc tgg     244
Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Ala Pro Ile Val Ile Trp
        60                  65                  70
aag tat aag tcg ttc tgt cgg gac cgt gtt gcc gac gcc ttc tcc cct     292
Lys Tyr Lys Ser Phe Cys Arg Asp Arg Val Ala Asp Ala Phe Ser Pro
    75                  80                  85
gcc agc gtg gac aac cag ctc aac gcc cag ctg gcg gct ggc aac ccc     340
Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro
90                  95                  100                 105
ggc tac aac ccc tat gtg gag tgc cag gac agc gta cgc act gtc agg     388
Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg Thr Val Arg
                110                 115                 120
gtg gtg gcc acc aaa cag ggc aat gct gtg acc ctg gga gac tac tac     436
Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr
            125                 130                 135
cag ggc agg aga atc acc atc aca gga aat gct ggc ctg acc ttc gag     484
Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Gly Leu Thr Phe Glu
        140                 145                 150
cag acg gcc tgg gga gac agt gga gtg tat tac tgc tcc gtg gtc tca     532
Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser Val Val Ser
    155                 160                 165
gcc caa gat ctg gat ggg aac aac gag gcg tac gca gag ctc att gtc     580
Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val
170                 175                 180                 185
ctt ggc agg acc tca gaa gcc cct gag ctc cta cct ggt ttt cgg gcg     628
Leu Gly Arg Thr Ser Glu Ala Pro Glu Leu Leu Pro Gly Phe Arg Ala
                190                 195                 200
ggg ccc ttg gaa gat tgg ctc ttt gtg gtc gtg gtc tgc ctg gca agc     676
Gly Pro Leu Glu Asp Trp Leu Phe Val Val Val Val Cys Leu Ala Ser
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        540                 545                 550
gaa gaa gaa gag gag ggc cac tat ccc cca gca cct ccg cct tac tct     1732
Glu Glu Glu Glu Glu Gly His Tyr Pro Pro Ala Pro Pro Pro Tyr Ser
    555                 560                 565
gag act gac tcg cag gcc tcg agg gag cgg agg atg aaa aag aat ttg     1780
Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg Met Lys Lys Asn Leu
570                 575                 580                 585
gcc ctg agt cgg gaa agt tta gtc gtc tga tcccacgttt tgttatgtag       1830
Ala Leu Ser Arg Glu Ser Leu Val Val  *
                590                 595
cttttatact tttttaattg gaatattgat gaaactcttc accaagccta ataaaa       1886

<210> 15
<211> 1829
<212> DNA
<213> Mus musculus

<400> 15
gcaccgtcgc tgctagacgg ccgcg atg gcg ccg gcg gcc agc gcg tgt gct        52
                            Met Ala Pro Ala Ala Ser Ala Cys Ala
                            1               5
ggg gcg cct ggc tcc cac ccg gcc acc acg atc ttc gtg tgt ctt ttt      100
Gly Ala Pro Gly Ser His Pro Ala Thr Thr Ile Phe Val Cys Leu Phe
10                  15                  20                  25
ctc atc att tac tgc cca gac cgt gcc agt gcc atc cag gtg acc gtg      148
Leu Ile Ile Tyr Cys Pro Asp Arg Ala Ser Ala Ile Gln Val Thr Val
                30                  35                  40
cct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg aca cta cac      196
Pro Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu His
            45                  50                  55
tgc acc tac cag atg agc aat acc ctc aca gcc cct atc gtg atc tgg      244
Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Ala Pro Ile Val Ile Trp
        60                  65                  70
aag tat aag tcg ttc tgt cgg gac cgt gtt gcc gac gcc ttc tcc cct      292
Lys Tyr Lys Ser Phe Cys Arg Asp Arg Val Ala Asp Ala Phe Ser Pro
    75                  80                  85
gcc agc gtg gac aac cag ctc aac gcc cag ctg gcg gct ggc aac ccc      340
Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro
90                  95                  100                 105
ggc tac aac ccc tat gtg gag tgc cag gac agc gta cgc act gtc agg      388
Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg Thr Val Arg
                110                 115                 120
gtg gtg gcc acc aaa cag ggc aat gct gtg acc ctg gga gac tac tac      436
Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr
            125                 130                 135
cag ggc agg aga atc acc atc aca gga aat gct ggc ctg acc ttc gag      484
Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Gly Leu Thr Phe Glu
        140                 145                 150
cag acg gcc tgg gga gac agt gga gtg tat tac tgc tcc gtg gtc tca      532
Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser Val Val Ser
    155                 160                 165
gcc caa gat ctg gat ggg aac aac gag gcg tac gca gag ctc att gtc      580
Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val
170                 175                 180                 185
ctt gat tgg ctc ttt gtg gtc gtg gtc tgc ctg gca agc ctc ctc ttc      628
Leu Asp Trp Leu Phe Val Val Val Val Cys Leu Ala Ser Leu Leu Phe
                190                 195                 200
ttc ctc ctc ctg ggc atc tgc tgg tgc cag tgc tgt ccc cac acc tgc      676
Phe Leu Leu Leu Gly Ile Cys Trp Cys Gln Cys Cys Pro His Thr Cys
            205                 210                 215
tgc tgc tat gtc aga tgt ccc tgc tgc cca gac aag tgc tgt tgc cct      724
Cys Cys Tyr Val Arg Cys Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro
        220                 225                 230
```

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gag gcc ctt tat gct gct ggc aaa gca gcc acc tca ggt gtg cca agc      772
Glu Ala Leu Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser
    235                 240                 245
atc tat gcc ccc agc atc tat acc cac ctc tct cct gcc aag act ccg      820
Ile Tyr Ala Pro Ser Ile Tyr Thr His Leu Ser Pro Ala Lys Thr Pro
250                 255                 260                 265
cca cct ccg cct gcc atg att ccc atg cgt cct ccc tat ggg tac cct      868
Pro Pro Pro Pro Ala Met Ile Pro Met Arg Pro Pro Tyr Gly Tyr Pro
                270                 275                 280
gga gac ttt gac agg acc agc tca gtt ggt ggc cac agc tcc cag gtg      916
Gly Asp Phe Asp Arg Thr Ser Ser Val Gly Gly His Ser Ser Gln Val
            285                 290                 295
ccc ctg ctg cgt gaa gtg gat ggg agc gta tct tca gaa gta cga agt      964
Pro Leu Leu Arg Glu Val Asp Gly Ser Val Ser Ser Glu Val Arg Ser
        300                 305                 310
ggc tac agg atc cag gct aac cag caa gat gac tcc atg agg gtc cta     1012
Gly Tyr Arg Ile Gln Ala Asn Gln Gln Asp Asp Ser Met Arg Val Leu
    315                 320                 325
tac tat atg gag aag gag cta gcc aac ttc gat cct tcc cgg cct ggc     1060
Tyr Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Pro Gly
330                 335                 340                 345
cct ccc aat ggc cga gtg gaa cgg gcc atg agt gaa gta acc tcc ctc     1108
Pro Pro Asn Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu
                350                 355                 360
cat gaa gat gac tgg cga tct cgg cct tcc agg gct cct gcc ctc aca     1156
His Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Ala Pro Ala Leu Thr
            365                 370                 375
ccc atc agg gat gag gag tgg aat cgc cac tcc cct cgg agt ccc aga     1204
Pro Ile Arg Asp Glu Glu Trp Asn Arg His Ser Pro Arg Ser Pro Arg
        380                 385                 390
aca tgg gag cag gaa ccc ctt caa gaa cag cca agg ggt ggt tgg ggg     1252
Thr Trp Glu Gln Glu Pro Leu Gln Glu Gln Pro Arg Gly Gly Trp Gly
    395                 400                 405
tct ggg cgg cct cgg gcc cgc tct gtg gat gct cta gat gac atc aac     1300
Ser Gly Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Ile Asn
410                 415                 420                 425
cgg cct ggc tcc act gaa tca gga agg tct tct ccc cca agt agt gga     1348
Arg Pro Gly Ser Thr Glu Ser Gly Arg Ser Ser Pro Pro Ser Ser Gly
                430                 435                 440
cgg aga ggg cgg gcc tat gca cct ccg aga agt cgc agc cgg gat gac     1396
Arg Arg Gly Arg Ala Tyr Ala Pro Pro Arg Ser Arg Ser Arg Asp Asp
            445                 450                 455
ctc tat gac ccc gac gat cct aga gac ttg cca cat tcc cga gat ccc     1444
Leu Tyr Asp Pro Asp Asp Pro Arg Asp Leu Pro His Ser Arg Asp Pro
        460                 465                 470
cac tat tat gat gat ttg agg tct agg gat cca cgt gct gac ccc aga     1492
His Tyr Tyr Asp Asp Leu Arg Ser Arg Asp Pro Arg Ala Asp Pro Arg
    475                 480                 485
tcc cgt cag cga tcc cac gat cct cgg gat gct ggc ttc agg tca cgg     1540
Ser Arg Gln Arg Ser His Asp Pro Arg Asp Ala Gly Phe Arg Ser Arg
490                 495                 500                 505
gac cct cag tat gat ggg cga ctc tta gaa gag gct tta aag aaa aaa     1588
Asp Pro Gln Tyr Asp Gly Arg Leu Leu Glu Glu Ala Leu Lys Lys Lys
                510                 515                 520
ggg gct ggg gag aga aga cgc gtt tac agg gag gaa gaa gaa gaa gaa     1636
Gly Ala Gly Glu Arg Arg Arg Val Tyr Arg Glu Glu Glu Glu Glu Glu
            525                 530                 535
gag gag ggc cac tat ccc cca gca cct ccg cct tac tct gag act gac     1684
Glu Glu Gly His Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp
        540                 545                 550
tcg cag gcc tcg agg gag cgg agg atg aaa aag aat ttg gcc ctg agt     1732
Ser Gln Ala Ser Arg Glu Arg Arg Met Lys Lys Asn Leu Ala Leu Ser
    555                 560                 565
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cgg gaa agt tta gtc gtc tga tcccacgttt tgttatgtag cttttatact        1783
Arg Glu Ser Leu Val Val  *
570                 575
tttttaattg gaatattgat gaaactcttc accaagccta ataaaa                 1829

<210> 16
<211> 1682
<212> DNA
<213> Mus musculus

<400> 16
gcaccgtcgc tgctagacgg ccgcg atg gcg ccg gcg gcc agc gcg tgt gct       52
                             Met Ala Pro Ala Ala Ser Ala Cys Ala
                             1               5
ggg gcg cct ggc tcc cac ccg gcc acc acg atc ttc gtg tgt ctt ttt      100
Gly Ala Pro Gly Ser His Pro Ala Thr Thr Ile Phe Val Cys Leu Phe
10                  15                  20                  25
ctc atc att tac tgc cca gac cgt gcc agt gcc atc cag gtg acc gtg      148
Leu Ile Ile Tyr Cys Pro Asp Arg Ala Ser Ala Ile Gln Val Thr Val
                30                  35                  40
cct gac ccc tac cac gta gtg atc ctg ttc cag cca gtg aca cta cac      196
Pro Asp Pro Tyr His Val Val Ile Leu Phe Gln Pro Val Thr Leu His
            45                  50                  55
tgc acc tac cag atg agc aat acc ctc aca gcc cct atc gtg atc tgg      244
Cys Thr Tyr Gln Met Ser Asn Thr Leu Thr Ala Pro Ile Val Ile Trp
        60                  65                  70
aag tat aag tcg ttc tgt cgg gac cgt gtt gcc gac gcc ttc tcc cct      292
Lys Tyr Lys Ser Phe Cys Arg Asp Arg Val Ala Asp Ala Phe Ser Pro
    75                  80                  85
gcc agc gtg gac aac cag ctc aac gcc cag ctg gcg gct ggc aac ccc      340
Ala Ser Val Asp Asn Gln Leu Asn Ala Gln Leu Ala Ala Gly Asn Pro
90                  95                  100                 105
ggc tac aac ccc tat gtg gag tgc cag gac agc gta cgc act gtc agg      388
Gly Tyr Asn Pro Tyr Val Glu Cys Gln Asp Ser Val Arg Thr Val Arg
                110                 115                 120
gtg gtg gcc acc aaa cag ggc aat gct gtg acc ctg gga gac tac tac      436
Val Val Ala Thr Lys Gln Gly Asn Ala Val Thr Leu Gly Asp Tyr Tyr
            125                 130                 135
cag ggc agg aga atc acc atc aca gga aat gct ggc ctg acc ttc gag      484
Gln Gly Arg Arg Ile Thr Ile Thr Gly Asn Ala Gly Leu Thr Phe Glu
        140                 145                 150
cag acg gcc tgg gga gac agt gga gtg tat tac tgc tcc gtg gtc tca      532
Gln Thr Ala Trp Gly Asp Ser Gly Val Tyr Tyr Cys Ser Val Val Ser
    155                 160                 165
gcc caa gat ctg gat ggg aac aac gag gcg tac gca gag ctc att gtc      580
Ala Gln Asp Leu Asp Gly Asn Asn Glu Ala Tyr Ala Glu Leu Ile Val
170                 175                 180                 185
ctt gtt tat gct gct ggc aaa gca gcc acc tca ggt gtg cca agc atc      628
Leu Val Tyr Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile
                190                 195                 200
tat gcc ccc agc atc tat acc cac ctc tct cct gcc aag act ccg cca      676
Tyr Ala Pro Ser Ile Tyr Thr His Leu Ser Pro Ala Lys Thr Pro Pro
            205                 210                 215
cct ccg cct gcc atg att ccc atg cgt cct ccc tat ggg tac cct gga      724
Pro Pro Pro Ala Met Ile Pro Met Arg Pro Pro Tyr Gly Tyr Pro Gly
        220                 225                 230
gac ttt gac agg acc agc tca gtt ggt ggc cac agc tcc cag gtg ccc      772
Asp Phe Asp Arg Thr Ser Ser Val Gly Gly His Ser Ser Gln Val Pro
    235                 240                 245
ctg ctg cgt gaa gtg gat ggg agc gta tct tca gaa gta cga agt ggc      820
Leu Leu Arg Glu Val Asp Gly Ser Val Ser Ser Glu Val Arg Ser Gly
250                 255                 260                 265
tac agg atc cag gct aac cag caa gat gac tcc atg agg gtc cta tac      868
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Tyr Arg Ile Gln Ala Asn Gln Gln Asp Asp Ser Met Arg Val Leu Tyr
                270                 275                 280
tat atg gag aag gag cta gcc aac ttc gat cct tcc cgg cct ggc cct      916
Tyr Met Glu Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Pro Gly Pro
            285                 290                 295
ccc aat ggc cga gtg gaa cgg gcc atg agt gaa gta acc tcc ctc cat      964
Pro Asn Gly Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu His
        300                 305                 310
gaa gat gac tgg cga tct cgg cct tcc agg gct cct gcc ctc aca ccc     1012
Glu Asp Asp Trp Arg Ser Arg Pro Ser Arg Ala Pro Ala Leu Thr Pro
    315                 320                 325
atc agg gat gag gag tgg aat cgc cac tcc cct cgg agt ccc aga aca     1060
Ile Arg Asp Glu Glu Trp Asn Arg His Ser Pro Arg Ser Pro Arg Thr
330                 335                 340                 345
tgg gag cag gaa ccc ctt caa gaa cag cca agg ggt ggt tgg ggg tct     1108
Trp Glu Gln Glu Pro Leu Gln Glu Gln Pro Arg Gly Gly Trp Gly Ser
                350                 355                 360
ggg cgg cct cgg gcc cgc tct gtg gat gct cta gat gac atc aac cgg     1156
Gly Arg Pro Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Ile Asn Arg
            365                 370                 375
cct ggc tcc act gaa tca gga agg tct tct ccc cca agt agt gga cgg     1204
Pro Gly Ser Thr Glu Ser Gly Arg Ser Ser Pro Pro Ser Ser Gly Arg
        380                 385                 390
aga ggg cgg gcc tat gca cct ccg aga agt cgc agc cgg gat gac ctc     1252
Arg Gly Arg Ala Tyr Ala Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu
    395                 400                 405
tat gac ccc gac gat cct aga gac ttg cca cat tcc cga gat ccc cac     1300
Tyr Asp Pro Asp Asp Pro Arg Asp Leu Pro His Ser Arg Asp Pro His
410                 415                 420                 425
tat tat gat gat ttg agg tct agg gat cca cgt gct gac ccc aga tcc     1348
Tyr Tyr Asp Asp Leu Arg Ser Arg Asp Pro Arg Ala Asp Pro Arg Ser
                430                 435                 440
cgt cag cga tcc cac gat cct cgg gat gct ggc ttc agg tca cgg gac     1396
Arg Gln Arg Ser His Asp Pro Arg Asp Ala Gly Phe Arg Ser Arg Asp
            445                 450                 455
cct cag tat gat ggg cga ctc tta gaa gag gct tta aag aaa aaa ggg     1444
Pro Gln Tyr Asp Gly Arg Leu Leu Glu Glu Ala Leu Lys Lys Lys Gly
        460                 465                 470
gct ggg gag aga aga cgc gtt tac agg gag gaa gaa gaa gaa gaa gag     1492
Ala Gly Glu Arg Arg Arg Val Tyr Arg Glu Glu Glu Glu Glu Glu Glu
    475                 480                 485
gag ggc cac tat ccc cca gca cct ccg cct tac tct gag act gac tcg     1540
Glu Gly His Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser
490                 495                 500                 505
cag gcc tcg agg gag cgg agg atg aaa aag aat ttg gcc ctg agt cgg     1588
Gln Ala Ser Arg Glu Arg Arg Met Lys Lys Asn Leu Ala Leu Ser Arg
                510                 515                 520
gaa agt tta gtc gtc tga tcccacgttt tgttatgtag cttttatact            1636
Glu Ser Leu Val Val  *
            525
tttttaattg gaatattgat gaaactcttc accaagccta ataaaa                  1682

<210> 17
<211> 594
<212> PRT
<213> Mus musculus

<400> 17
Met Ala Pro Ala Ala Ser Ala Cys Ala Gly Ala Pro Gly Ser His Pro
1               5                   10                  15
Ala Thr Thr Ile Phe Val Cys Leu Phe Leu Ile Ile Tyr Cys Pro Asp
            20                  25                  30
Arg Ala Ser Ala Ile Gln Val Thr Val Pro Asp Pro Tyr His Val Val
```

```
        35                  40                  45
Ile Leu Phe Gln Pro Val Thr Leu His Cys Thr Tyr Gln Met Ser Asn
    50                  55                  60
Thr Leu Thr Ala Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65                  70                  75                  80
Asp Arg Val Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
                85                  90                  95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
            100                 105                 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
        115                 120                 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
    130                 135                 140
Thr Gly Asn Ala Gly Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145                 150                 155                 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
                165                 170                 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Gly Arg Thr Ser Glu Ala
            180                 185                 190
Pro Glu Leu Leu Pro Gly Phe Arg Ala Gly Pro Leu Glu Asp Trp Leu
        195                 200                 205
Phe Val Val Val Val Cys Leu Ala Ser Leu Leu Phe Phe Leu Leu Leu
    210                 215                 220
Gly Ile Cys Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr Val
225                 230                 235                 240
Arg Cys Pro Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr
                245                 250                 255
Ala Ala Gly Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro
            260                 265                 270
Ser Ile Tyr Thr His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro
        275                 280                 285
Ala Met Ile Pro Met Arg Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp
    290                 295                 300
Arg Thr Ser Ser Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg
305                 310                 315                 320
Glu Val Asp Gly Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile
                325                 330                 335
Gln Ala Asn Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu
            340                 345                 350
Lys Glu Leu Ala Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly
        355                 360                 365
Arg Val Glu Arg Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp
    370                 375                 380
Trp Arg Ser Arg Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp
385                 390                 395                 400
Glu Glu Trp Asn Arg His Ser Pro Arg Ser Pro Arg Thr Trp Glu Gln
                405                 410                 415
Glu Pro Leu Gln Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro
            420                 425                 430
Arg Ala Arg Ser Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser
        435                 440                 445
Thr Glu Ser Gly Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg
    450                 455                 460
Ala Tyr Ala Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro
465                 470                 475                 480
Asp Asp Pro Arg Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp
                485                 490                 495
Asp Leu Arg Ser Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg
            500                 505                 510
Ser His Asp Pro Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr
        515                 520                 525
Asp Gly Arg Leu Leu Glu Glu Ala Leu Lys Lys Lys Gly Ala Gly Glu
    530                 535                 540
```

```
Arg Arg Arg Val Tyr Arg Glu Glu Glu Glu Glu Glu Glu Glu Gly His
545                 550                 555                 560
Tyr Pro Pro Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser
                565                 570                 575
Arg Glu Arg Arg Met Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu
            580                 585                 590
Val Val
```

<210> 18
<211> 575
<212> PRT
<213> Mus musculus

<400> 18

```
Met Ala Pro Ala Ala Ser Ala Cys Ala Gly Ala Pro Gly Ser His Pro
1               5                  10                  15
Ala Thr Thr Ile Phe Val Cys Leu Phe Leu Ile Ile Tyr Cys Pro Asp
            20                  25                  30
Arg Ala Ser Ala Ile Gln Val Thr Val Pro Asp Pro Tyr His Val Val
        35                  40                  45
Ile Leu Phe Gln Pro Val Thr Leu His Cys Thr Tyr Gln Met Ser Asn
    50                  55                  60
Thr Leu Thr Ala Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65                  70                  75                  80
Asp Arg Val Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
                85                  90                  95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
            100                 105                 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
        115                 120                 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
    130                 135                 140
Thr Gly Asn Ala Gly Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145                 150                 155                 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
                165                 170                 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Asp Trp Leu Phe Val Val
            180                 185                 190
Val Val Cys Leu Ala Ser Leu Leu Phe Phe Leu Leu Leu Gly Ile Cys
        195                 200                 205
Trp Cys Gln Cys Cys Pro His Thr Cys Cys Cys Tyr Val Arg Cys Pro
    210                 215                 220
Cys Cys Pro Asp Lys Cys Cys Cys Pro Glu Ala Leu Tyr Ala Ala Gly
225                 230                 235                 240
Lys Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr
                245                 250                 255
Thr His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro Ala Met Ile
            260                 265                 270
Pro Met Arg Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp Arg Thr Ser
        275                 280                 285
Ser Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg Glu Val Asp
    290                 295                 300
Gly Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn
305                 310                 315                 320
Gln Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu
                325                 330                 335
Ala Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly Arg Val Glu
            340                 345                 350
Arg Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp Trp Arg Ser
        355                 360                 365
Arg Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp
    370                 375                 380
Asn Arg His Ser Pro Arg Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu
```

385 390 395 400
Gln Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg
405 410 415
Ser Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser
420 425 430
Gly Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala
435 440 445
Pro Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro
450 455 460
Arg Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp Asp Leu Arg
465 470 475 480
Ser Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg Ser His Asp
485 490 495
Pro Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg
500 505 510
Leu Leu Glu Glu Ala Leu Lys Lys Lys Gly Ala Gly Glu Arg Arg Arg
515 520 525
Val Tyr Arg Glu Glu Glu Glu Glu Glu Glu Glu Glu Gly His Tyr Pro Pro
530 535 540
Ala Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg
545 550 555 560
Arg Met Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val Val
565 570 575

<210> 19
<211> 526
<212> PRT
<213> Mus musculus

<400> 19
Met Ala Pro Ala Ala Ser Ala Cys Ala Gly Ala Pro Gly Ser His Pro
1 5 10 15
Ala Thr Thr Ile Phe Val Cys Leu Phe Leu Ile Ile Tyr Cys Pro Asp
20 25 30
Arg Ala Ser Ala Ile Gln Val Thr Val Pro Asp Pro Tyr His Val Val
35 40 45
Ile Leu Phe Gln Pro Val Thr Leu His Cys Thr Tyr Gln Met Ser Asn
50 55 60
Thr Leu Thr Ala Pro Ile Val Ile Trp Lys Tyr Lys Ser Phe Cys Arg
65 70 75 80
Asp Arg Val Ala Asp Ala Phe Ser Pro Ala Ser Val Asp Asn Gln Leu
85 90 95
Asn Ala Gln Leu Ala Ala Gly Asn Pro Gly Tyr Asn Pro Tyr Val Glu
100 105 110
Cys Gln Asp Ser Val Arg Thr Val Arg Val Val Ala Thr Lys Gln Gly
115 120 125
Asn Ala Val Thr Leu Gly Asp Tyr Tyr Gln Gly Arg Arg Ile Thr Ile
130 135 140
Thr Gly Asn Ala Gly Leu Thr Phe Glu Gln Thr Ala Trp Gly Asp Ser
145 150 155 160
Gly Val Tyr Tyr Cys Ser Val Val Ser Ala Gln Asp Leu Asp Gly Asn
165 170 175
Asn Glu Ala Tyr Ala Glu Leu Ile Val Leu Val Tyr Ala Ala Gly Lys
180 185 190
Ala Ala Thr Ser Gly Val Pro Ser Ile Tyr Ala Pro Ser Ile Tyr Thr
195 200 205
His Leu Ser Pro Ala Lys Thr Pro Pro Pro Pro Pro Ala Met Ile Pro
210 215 220
Met Arg Pro Pro Tyr Gly Tyr Pro Gly Asp Phe Asp Arg Thr Ser Ser
225 230 235 240
Val Gly Gly His Ser Ser Gln Val Pro Leu Leu Arg Glu Val Asp Gly
245 250 255
Ser Val Ser Ser Glu Val Arg Ser Gly Tyr Arg Ile Gln Ala Asn Gln

```
            260                 265                 270
Gln Asp Asp Ser Met Arg Val Leu Tyr Tyr Met Glu Lys Glu Leu Ala
        275                 280                 285
Asn Phe Asp Pro Ser Arg Pro Gly Pro Pro Asn Gly Arg Val Glu Arg
    290                 295                 300
Ala Met Ser Glu Val Thr Ser Leu His Glu Asp Asp Trp Arg Ser Arg
305                 310                 315                 320
Pro Ser Arg Ala Pro Ala Leu Thr Pro Ile Arg Asp Glu Glu Trp Asn
                325                 330                 335
Arg His Ser Pro Arg Ser Pro Arg Thr Trp Glu Gln Glu Pro Leu Gln
            340                 345                 350
Glu Gln Pro Arg Gly Gly Trp Gly Ser Gly Arg Pro Arg Ala Arg Ser
        355                 360                 365
Val Asp Ala Leu Asp Asp Ile Asn Arg Pro Gly Ser Thr Glu Ser Gly
    370                 375                 380
Arg Ser Ser Pro Pro Ser Ser Gly Arg Arg Gly Arg Ala Tyr Ala Pro
385                 390                 395                 400
Pro Arg Ser Arg Ser Arg Asp Asp Leu Tyr Asp Pro Asp Asp Pro Arg
                405                 410                 415
Asp Leu Pro His Ser Arg Asp Pro His Tyr Tyr Asp Asp Leu Arg Ser
            420                 425                 430
Arg Asp Pro Arg Ala Asp Pro Arg Ser Arg Gln Arg Ser His Asp Pro
        435                 440                 445
Arg Asp Ala Gly Phe Arg Ser Arg Asp Pro Gln Tyr Asp Gly Arg Leu
    450                 455                 460
Leu Glu Glu Ala Leu Lys Lys Lys Gly Ala Gly Glu Arg Arg Arg Val
465                 470                 475                 480
Tyr Arg Glu Glu Glu Glu Glu Glu Glu Glu Gly His Tyr Pro Pro Ala
                485                 490                 495
Pro Pro Pro Tyr Ser Glu Thr Asp Ser Gln Ala Ser Arg Glu Arg Arg
            500                 505                 510
Met Lys Lys Asn Leu Ala Leu Ser Arg Glu Ser Leu Val Val
        515                 520                 525
```

<210> 20
<211> 18
<212> DNA
<213> Homo Sapiens

<220>
<221> misc_binding
<222> 1..18
<223> sequencing oligonucleotide PrimerPU

<400> 20
tgtaaaacga cggccagt 18

<210> 21
<211> 18
<212> DNA
<213> Homo Sapiens

<220>
<221> misc_binding
<222> 1..18
<223> sequencing oligonucleotide PrimerRP

<400> 21
caggaaacag ctatgacc 18

<210> 22
<211> 20
<212> DNA

<213> Artificial Sequence

<220>
<223> oligonucleotide sense primer

<400> 22
ctacaacccc tacgtcgagt 20

<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide anti sense primer

<400> 23
aggcggagat cgccagtcgt 20

<210> 24
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide sense primer

<400> 24
cctttgtcca cgtcgtttac gctc 24

<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide anti sense primer

<400> 25
tcacagcgtt gccctgcttg 20

<210> 26
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide sense primer

<400> 26
ttactgctcc gtggtctcag c 21

<210> 27
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide anti sense primer

<400> 27
agctactcct gtcaacgtct cc 22

<210> 28
<211> 167
<212> PRT
<213> Bos taurus

<400> 28
Met Arg Cys Gly Pro Leu Tyr Arg Phe Leu Trp Leu Trp Pro Tyr Leu
1 5 10 15
Ser Tyr Val Glu Ala Val Pro Ile Arg Lys Val Gln Asp Asp Thr Lys
20 25 30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
35 40 45
Gln Ser Val Ser Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
50 55 60
Gly Leu His Pro Leu Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala
65 70 75 80
Ile Tyr Gln Gln Ile Leu Thr Ser Leu Pro Ser Arg Asn Val Val Gln
85 90 95
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
100 105 110
Ala Ser Lys Ser Cys Pro Leu Pro Gln Val Arg Ala Leu Glu Ser Leu
115 120 125
Glu Ser Leu Gly Val Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
130 135 140
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Arg Gln
145 150 155 160
Leu Asp Leu Ser Pro Gly Cys
165

<210> 29
<211> 146
<212> PRT
<213> Canis familiaris

<400> 29
Val Pro Ile Arg Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1 5 10 15
Ile Val Ala Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
20 25 30
Lys Gln Arg Val Ala Gly Leu Asp Phe Ile Pro Gly Leu Gln Pro Val
35 40 45
Leu Ser Leu Ser Arg Met Asp Gln Thr Leu Ala Ile Tyr Gln Gln Ile
50 55 60
Leu Asn Ser Leu His Ser Arg Asn Val Val Gln Ile Ser Asn Asp Leu
65 70 75 80
Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Ser Ser Lys Ser Cys
85 90 95
Pro Leu Pro Arg Ala Arg Gly Leu Glu Thr Phe Glu Ser Leu Gly Gly
100 105 110
Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
115 120 125
Leu Gln Ala Ala Leu Gln Asp Met Leu Arg Arg Leu Asp Leu Ser Pro
130 135 140
Gly Cys
145

<210> 30
<211> 163
<212> PRT
<213> Gallus gallus

<400> 30
Met Cys Trp Arg Pro Leu Cys Arg Leu Trp Ser Tyr Leu Val Tyr Val

```
1               5                   10                  15
Gln Ala Val Pro Cys Gln Ile Phe Gln Asp Asp Thr Lys Thr Leu Ile
            20                  25                  30
Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Ser Val Ser
        35                  40                  45
Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro
    50                  55                  60
Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln
65                  70                  75                  80
Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln Ile Ala Asn Asp
                85                  90                  95
Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Phe Ser Lys Ser
            100                 105                 110
Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser Leu Asp
        115                 120                 125
Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser
    130                 135                 140
Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Ile Ser
145                 150                 155                 160
Pro Glu Cys
```

<210> 31
<211> 146
<212> PRT
<213> Gorilla gorilla

<400> 31
```
Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1               5                   10                  15
Ile Val Thr Arg Ile Ser Asp Ile Ser His Thr Gln Ser Val Ser Ser
            20                  25                  30
Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Ile
        35                  40                  45
Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile
    50                  55                  60
Leu Thr Ser Met Pro Ser Arg Asn Met Ile Gln Ile Ser Asn Asp Leu
65                  70                  75                  80
Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys
                85                  90                  95
His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly
            100                 105                 110
Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
        115                 120                 125
Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
    130                 135                 140
Gly Cys
145
```

<210> 32
<211> 167
<212> PRT
<213> Homo sapiens

<400> 32
```
Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu
1               5                   10                  15
Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
            20                  25                  30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
        35                  40                  45
Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro
    50                  55                  60
Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala
```

65 70 75 80
Val Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln
85 90 95
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala
100 105 110
Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu
115 120 125
Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val
130 135 140
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln
145 150 155 160
Leu Asp Leu Ser Pro Gly Cys
165

<210> 33
<211> 167
<212> PRT
<213> Macaca mulatta

<400> 33
Met Tyr Trp Arg Thr Leu Trp Gly Phe Leu Trp Leu Trp Pro Tyr Leu
1 5 10 15
Phe Tyr Ile Gln Ala Val Pro Ile Gln Lys Val Gln Ser Asp Thr Lys
20 25 30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
35 40 45
Gln Ser Val Ser Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
50 55 60
Gly Leu His Pro Val Leu Thr Leu Ser Gln Met Asp Gln Thr Leu Ala
65 70 75 80
Ile Tyr Gln Gln Ile Leu Ile Asn Leu Pro Ser Arg Asn Val Ile Gln
85 90 95
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
100 105 110
Phe Ser Lys Ser Cys His Leu Pro Leu Ala Ser Gly Leu Glu Thr Leu
115 120 125
Glu Ser Leu Gly Asp Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
130 135 140
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln
145 150 155 160
Leu Asp Leu Ser Pro Gly Cys
165

<210> 34
<211> 167
<212> PRT
<213> Mus musculus

<400> 34
Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu
1 5 10 15
Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
20 25 30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
35 40 45
Gln Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
50 55 60
Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala
65 70 75 80
Val Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln
85 90 95
Ile Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
100 105 110

```
Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro
        115                 120                 125
Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
    130                 135                 140
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln
145                 150                 155                 160
Leu Asp Val Ser Pro Glu Cys
                165
```

<210> 35
<211> 146
<212> PRT
<213> Ovus aries

<400> 35
```
Val Pro Ile Arg Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1               5                  10                  15
Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
            20                  25                  30
Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Leu
        35                  40                  45
Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Ile Tyr Gln Gln Ile
    50                  55                  60
Leu Ala Ser Leu Pro Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu
65                  70                  75                  80
Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Ala Ser Lys Ser Cys
                85                  90                  95
Pro Leu Pro Gln Val Arg Ala Leu Glu Ser Leu Glu Ser Leu Gly Val
            100                 105                 110
Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
        115                 120                 125
Leu Gln Gly Ser Leu Gln Asp Met Leu Arg Gln Leu Asp Leu Ser Pro
    130                 135                 140
Gly Cys
145
```

<210> 36
<211> 146
<212> PRT
<213> Pan troglodytes

<400> 36
```
Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1               5                  10                  15
Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
            20                  25                  30
Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Ile
        35                  40                  45
Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile
    50                  55                  60
Leu Thr Ser Met Pro Ser Arg Asn Met Ile Gln Ile Ser Asn Asp Leu
65                  70                  75                  80
Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys
                85                  90                  95
His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly
            100                 105                 110
Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
        115                 120                 125
Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
    130                 135                 140
Gly Cys
145
```

<210> 37
<211> 146
<212> PRT
<213> Pongo pygmaeus

<400> 37
Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1 5 10 15
Val Ile Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
20 25 30
Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Ile
35 40 45
Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile
50 55 60
Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu
65 70 75 80
Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys
85 90 95
His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Arg Leu Gly Gly
100 105 110
Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
115 120 125
Leu Gln Arg Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
130 135 140
Gly Cys
145

<210> 38
<211> 167
<212> PRT
<213> Rattus norvegicus

<400> 38
Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu
1 5 10 15
Ser Tyr Val Gln Ala Val Pro Ile His Lys Val Gln Asp Asp Thr Lys
20 25 30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
35 40 45
Gln Ser Val Ser Ala Arg Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
50 55 60
Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala
65 70 75 80
Val Tyr Gln Gln Ile Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln
85 90 95
Ile Ala His Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
100 105 110
Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Arg Gly Leu Gln Lys Pro
115 120 125
Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
130 135 140
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln
145 150 155 160
Leu Asp Leu Ser Pro Glu Cys
165

<210> 39
<211> 167
<212> PRT
<213> Sus scrofa

<400> 39
Met Arg Cys Gly Pro Leu Cys Arg Phe Leu Trp Leu Trp Pro Tyr Leu

```
1               5                   10                  15
Ser Tyr Val Glu Ala Val Pro Ile Trp Arg Val Gln Asp Asp Thr Lys
            20                  25                  30
Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Ser Asp Ile Ser His Met
        35                  40                  45
Gln Ser Val Ser Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
    50                  55                  60
Gly Leu His Pro Val Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala
65                  70                  75                  80
Ile Tyr Gln Gln Ile Leu Thr Ser Leu Pro Ser Arg Asn Val Ile Gln
                85                  90                  95
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
            100                 105                 110
Ser Ser Lys Ser Cys Pro Leu Pro Gln Ala Arg Ala Leu Glu Thr Leu
        115                 120                 125
Glu Ser Leu Gly Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
    130                 135                 140
Val Ala Leu Ser Arg Leu Gln Gly Ala Leu Gln Asp Met Leu Arg Gln
145                 150                 155                 160
Leu Asp Leu Ser Pro Gly Cys
                165
```

<210> 40
<211> 4
<212> PRT
<213> Homo sapiens

<400> 40
Glu Thr Leu Asp
1

<210> 41
<211> 4
<212> PRT
<213> Mus musculus

<400> 41
Gln Lys Pro Glu
1

<210> 42
<211> 6
<212> PRT
<213> Homo sapiens

<400> 42
Leu Asp Ser Leu Gly Gly
1   5

<210> 43
<211> 4
<212> PRT
<213> Homo sapiens

<400> 43
Glu Lys Leu Glu
1

<210> 44
<211> 4
<212> PRT
<213> Homo sapiens

<400> 44
Glu Lys Pro Glu
1

<210> 45
<211> 4
<212> PRT
<213> Homo sapiens

<400> 45
Glu Lys Pro Asp
1

<210> 46
<211> 5
<212> PRT
<213> Homo sapiens

<400> 46
Thr Pro Asp Ser Leu
1 5

<210> 47
<211> 9
<212> PRT
<213> Homo sapiens

<400> 47
Gly Leu Gln Thr Leu Asp Ser Leu Gly
1 5

<210> 48
<211> 5
<212> PRT
<213> Homo sapiens

<400> 48
Gly Gly Val Leu Glu
1 5

<210> 49
<211> 6
<212> PRT
<213> Homo sapiens

<400> 49
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<210> 51
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<400> 53
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<210> 54
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His Leu Pro Trp Ala Ser
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<210> 55
<211> 22
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<210> 56
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<210> 57
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Gly Val Leu Glu Ala Ser
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<400> 58
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Leu Glu

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<400> 59
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Gly Tyr Ser Thr Glu
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<400> 61
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1 5 10

<210> 62
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Glu Val Val Ala Leu Ser
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<210> 63
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Pro Glu Ser Leu Asp Gly
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<210> 67
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Gly Val Leu Glu Ala Ser
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<210> 68
<211> 18
<212> PRT
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Leu Glu

<210> 69
<211> 14
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Ser Leu Tyr Ser Thr Glu
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1 5 10

<210> 72
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Glu Val Val Ala Leu Ser
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cctactccaa gtcmgtcctg ttgcatt 27

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<210> 80
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<400> 80
ccagatgacg atgggttgc 19

<210> 81
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<220>
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<400> 81
accctgccct gwcctaccag atgac 25

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<400> 82
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tttcgcgc 68

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<210> 84
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<220>
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agtcttctat gggccccgc 19

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caccgactcg agamtggacc aaaagtc 27

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acgcagagct catcgtcctt 20

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gatgcccagg aggaggaaga 20

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<220>
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<400> 89
caacaccata ckgaccgacg gaa 23

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<220>
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<400> 102
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<400> 103
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<210> 104
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<400> 104
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<400> 105
ccgggagtgc gcaggggta 20

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<400> 106
gtggctgcac aaggtcgcc 19